Development of a therapeutic vaccination strategy against cervical neoplasia

The printing of this thesis was financially supported by:
Bayer HealthCare, GlaxoSmithKline, Graduate School for Drug Exploration (GUIDE), Sanofi Pasteur MSD, University of Groningen, Wyeth.

ISBN: 978-90-367-3298-7
ISBN: 978-90-367-3320-5 (electronic version)

Cover design:
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Page layout: P. van der Sijde, Groningen, The Netherlands
Printed by: Ponsen & Looijen, Wageningen

RIJKSUNIVERSITEIT GRONINGEN

Development of a therapeutic vaccination strategy against cervical neoplasia

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
woensdag 6 februari 2008
om 13.15 uur

door

Annelies Riezebos-Brilman

geboren op 3 juni 1975
te Nijmegen

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Voor David, Ruth & Hannah,
ik hou van jullie

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CHAPTER 1

General introduction

CERVICAL CANCER AND HUMAN PAPILLOMAVIRUS

Cervical cancer is the second most common cancer among women worldwide. The number of new cases diagnosed annually is estimated around 500.000, with 250.000 deaths. Over 80% of new cervical cancer cases occur in developing countries, where neither population-based routine screening nor adequate treatment is available. The highest incidence rates are observed in sub-Saharan Africa, Melanesia, Latin America and the Caribbean, South-Central Asia, and South-East Asia.[1] In the Netherlands, despite excellent screening and treatment possibilities, 600-700 women are diagnosed with cervical cancer annually, and 200-250 patients die from the disease.[2;3]

The majority of cases of cervical cancer are squamous cell carcinomas, followed by adenocarcinomas and adenosquamous carcinomas.[4] Cervical cancer develops from pre-existing non-invasive premalignant lesions, so-called cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL). These lesions are classified histologically on the basis of progressive atypia of epithelial cells. CIN I relates to mild dysplasia, CIN II to moderate dysplasia, and CIN III to both severe dysplasia and carcinoma *in situ*. CIN I is also classified as low-grade SIL and CIN II and CIN III as high-grade SIL.[5] (Figure 1)

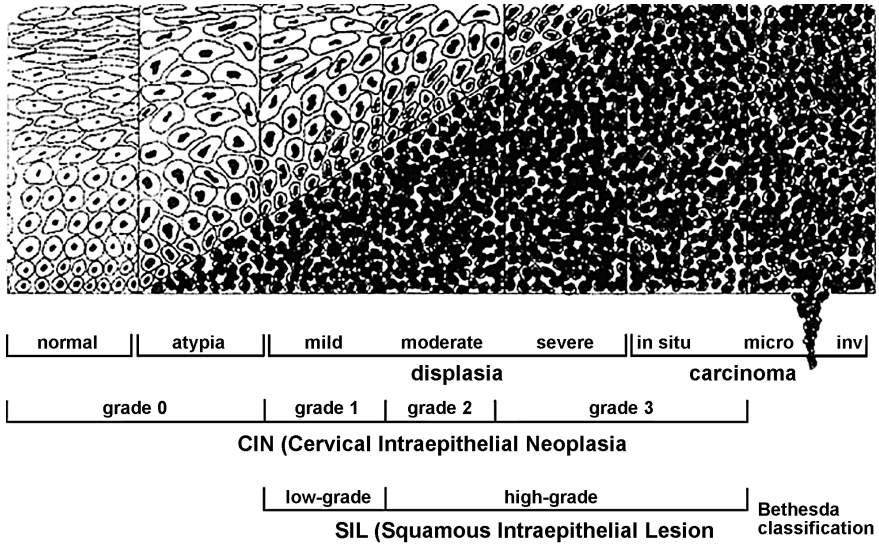

Figure 1. Schematic overview of the morphological alterations of normal cervical epithelial cells toward invasive cervical cancer. (Reproduced, with permission, from Snijders *et al.* J. Pathol 2006; 208: 152-164)

Epidemiologic classification of HPV types

Recently, cervical cancer has been recognized by the World Health Organization (WHO) as the first cancer to be 100% attributable to infection with a virus, the Human Papillomavirus (HPV).[6] Over 100 HPV types have been characterized molecularly and about 30 to 40 types are able to infect the mucosa of the genital tract.[7] Fifteen types are considered to be high-risk types in terms of oncogenic potential (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82). Three HPV types are classified as probable high-risk types (26, 53, and 66), and 12 types are classified as low-risk (6, 11, 40,42, 44, 54, 61, 70, 72, 81, and CP6108).[8] Infection with low-risk HPV types can cause benign lesions of the anogenital areas known as condylomata acuminata (genital warts) or low-grade squamous intraepithelial lesions of the cervix (CIN I and CIN II), and recurrent respiratory papillomatosis.[4]

Persistent infection with high-risk HPVs represents a necessary cause of cervical cancer. High-risk HPV DNA is detected in virtually all cases (>99%) of cervical cancers, and in up to 94% of women with CIN lesions.[9-11] The eight most common high-risk types (16, 18, 45, 31, 33, 52, 58, and 35) account for 89% of all cervical cancer cases worldwide. HPV 16 is the cause of about 54% of invasive cervical cancers and HPV 18 is the cause of about 17%.[8;12] (Figure 2) To a lesser extent, these high-risk HPV types are also found to be a cause of a substantial proportion of other anogenital neoplasia (penile, vaginal, vulvae, and anal) and oral squamous cell carcinomas.[1]

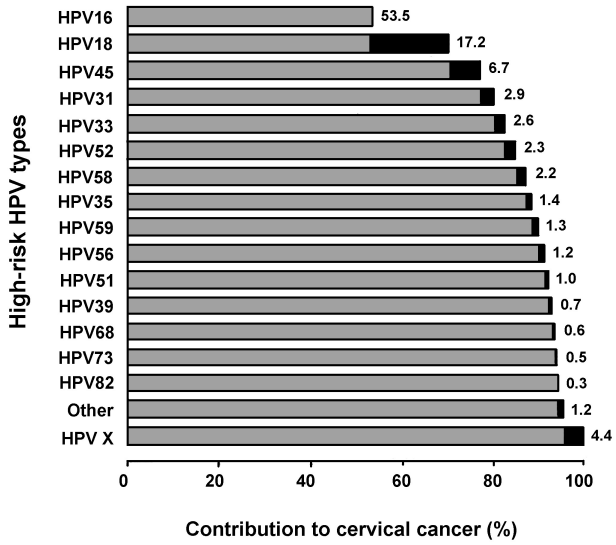

Figure 2. The cumulative frequency of high-risk HPV types present in cervical cancer (Reproduced, with permission, from Munoz *et al.* Int. J. Cancer 2004; 111(2): 278-85)

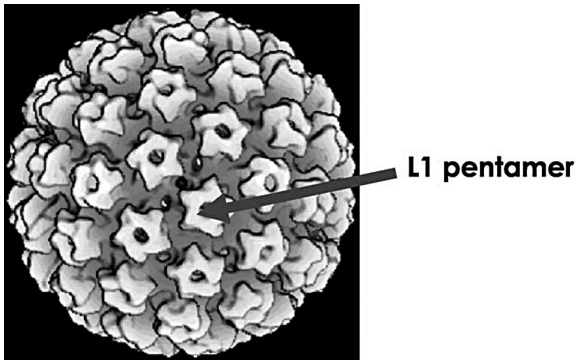

Figure 3. A model of the papillomavirus capsid. (Reproduced, with permission from Elsevier, from Stanley *et al.* Vaccine 2006;24 Suppl 3: S106-S113)

HPV biology

The Human Papillomavirus belongs to the family *Papovaviridae*. Papillomaviruses are small, nonenveloped, icosahedral DNA viruses that replicate in the nucleus of squamous epithelial cells. (Figure 3) The virion is composed of a double-stranded circular 8000-basepair DNA genome surrounded by an icosahedral protein capsid of about 55 nm in diameter. The HPV genome is divided into an early region, encoding genes required for DNA replication and cellular transformation (E1, E2, E4, E5, E6, and E7) and a late region, encoding the two viral capsid proteins L1 and L2. Papillomaviruses are able to infect the basal cells of the epithelium when the integrity of the epithelium is compromised by a micro-abrasion or other traumas. The viral genome then becomes established in the basal cells as an episome, and progeny virions are generated. In these basal layers of stratified epithelium, viral early proteins are produced in undifferentiated keratinocytes. Infected daughter cells then begin to migrate up and differentiate in the outer layers of the epithelium. In these outer layers late proteins are produced and capsids are formed. Subsequently, virions are shed into the genital tract from desquamated epithelial cells.[10;13] The viral DNA is mostly in an episomal form in low-grade lesions, but it is integrated into the host cell chromosome in high-grade lesions and cancer.[14] Integration disrupts the E2 open reading frame (ORF), resulting in deregulation of E6 and E7 gene products, which may lead to cellular transformation.[15-17]

The E6 and E7 genes are both required and cooperate to induce immortalization of human genital keratinocytes. The E7 protein functions in cellular transformation through interactions with the retinoblastoma protein (pRB) and the other pRB-related 'pocket proteins', which leads to the expression of proteins necessary for DNA replication. The viral E6 protein complements the role of E7, and prevents the induction of apoptosis. E6 is able to interact with many cellular factors. The

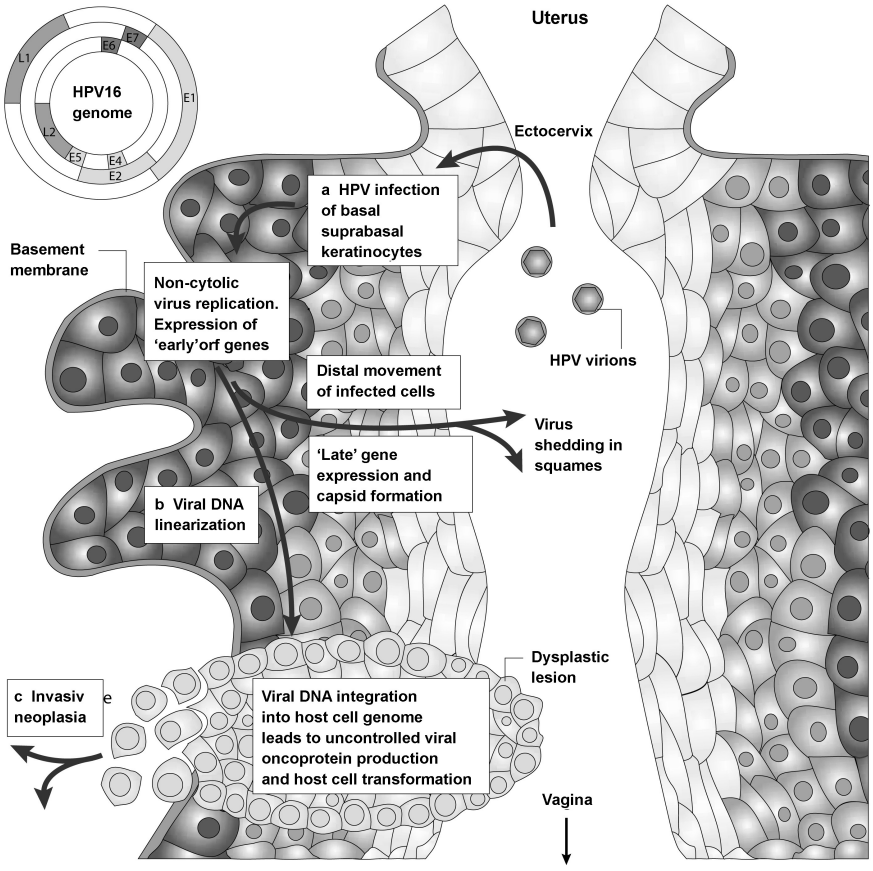

Figure 4. The biology of HPV infection
(a) Human papillomavirus (HPV) virions infect basal cells of stratified mucosal epithelium at the junction between the vagina and ectocervix. Limited viral replication is accompanied by expression of 'early' proteins E1 and E2 in the basal layers. In more distal layers, E6 and E7 are expressed. These proteins promote cell proliferation and delay differentiation. As infected cells differentiate into squamous cells, the E4 protein, and the late proteins L1 and L2 (which form the capsid), are expressed. Viral capsids are shed into the genital tract within desquamated epithelial cells. (b) Rarely, the DNA of oncogenic HPVs linearizes randomly and integrates into the host cell genome. When the break occurs in the E2 region, the lifting of E2-mediated transcriptional repression of E6 and E7 oncogenes predisposes infected cells to cellular transformation (dysplasia). (c) Invasive tumor ruptures the basement membrane and invades the sub-epidermal tissue. Inset shows the HPV genome. The ~8 kilobase double-stranded DNA genome encodes eight open reading frames (ORF): six 'early' proteins (E1, E2, E4, E5, E6 and E7), and two 'late' proteins (L1 and L2) L1 and L2 encode capsid proteins; E1 and E2 are involved in DNA replication and transcriptional control; E4 might aggregate cytokeratins or form intermediate-filament-like structures; E5 inhibits acidification of endosomes and down regulates major histocompatibility complex (MHC) class I expression. (Reproduced, with permission from Macmillan Publishers Ltd, from Tindle *et al.* Nat Rev Cancer 2002;2(1):59-64)

major target of E6 is p53, with E6 binding to p53 resulting in inactivation of p53-mediated growth suppression and/or apoptosis. Continued production of these two oncoproteins is required for the maintenance of the transformed phenotype of (pre)malignant cells.[18;19] (Figure 4)

Prevalence of HPV infection and risk-factors

HPV infections are the most commonly diagnosed sexually transmitted diseases today. Acquisition of HPV occurs soon after sexual initiation.[20] Estimates of the population prevalence of HPV infection among women around the world range from 2% to 44%. The prevalence of HPV infection is highest among young sexually active women and appears to drop with increasing age.[21;22] It is estimated that it takes on average 12-15 years before a persistent infection with high-risk HPV develops into cervical carcinoma.[23;24] Progression from clinically detectable infection to invasive cervical carcinoma occurs in less than 1% of the infected women.

At first it was thought that cervical cancer would always evolve from infected normal cervical epithelium via consecutive CIN I, CIN II, and CIN III lesions. However, most CIN I and CIN II lesions are manifestations of a normal productive infection, and in the majority of cases are caused by low-risk HPV types. Over 90% of these lesions will regress spontaneously.[5] Recently, Winer *et al.*[25] showed that many of the clinically relevant CIN II/III lesions may be rapidly induced within 2 years following infection with high-risk HPV. However, notably, also in these cases only a minority of the lesions will develop into invasive cervical cancer. The mean age of women with CIN III is approximately 28 years, while the mean age of women with invasive cervical cancer is approximately 50 years. Given this long precancerous state and the predominating transient nature of the HPV infection it is suggested that other factors also play a role in the carcinogenic process.

Studies reviewing the risk of CIN III or cervical cancer among HPV-positive women have classified smoking as a co-risk factor.[26] This has been confirmed in recent prospective studies.[27;28] The long-term use of oral contraceptive in relation to the development of high-grade cervical lesions among HPV-positive women is more ambivalent.[29] No association between oral contraceptive use and CIN III in HPV-positive women could be found in several prospective studies.[30;31] Co-infection with *Chlamydia trachomatis* and herpes simplex virus type-2, immunosuppression, and certain dietary deficiencies have been suggested as other probable co-factors. However the currently available evidence for these co-factors is not convincing.[26;29;32]

Current screening and treatment options

In the developed world, as a result of intensive screening programs, cervical cancer or preceding CIN lesions are usually detected at an early stage. Cytomorphological examination of cervical smears is the most widely applied screening-method for cervical cancer and it precursors. Cervical smears are classified according to a modified Papanicolaou system or the Bethesda classification system. Other methods used for screening are liquid based cytology, HPV testing, and combined testing.

The most common techniques for treatment of CIN II/III are loop electrosurgical excision procedure (LEEP), cryotherapy, carbon dioxide laser ablation or conization (cold knife or laser). These techniques have been shown equally effective, averaging approximately 90% cure, and an overall rate of recurrent or persistent disease of 5 to 17%.[33]

With cervical cancer, clinical stage is the most important prognostic parameter and, therefore, determines the choice of treatment. The International Federation of Gynecology & Obstetrics (FIGO) has established criteria for clinical staging of cervical cancer in which tumor-size and involvement of the vagina and parametrium are estimated.[34] Microinvasive cancer (FIGO stage IA) can be treated by LEEP or conization to maintain fertility or by simple hysterectomy. Therapy options for early stage cervical cancer (FIGO stage IB1, nonbulky IIA) are radical hysterectomy with or without adjuvant therapy, definitive radiation therapy or radical trachelectomy. Randomized trials suggest that survival is similar with radical hysterectomy with or without postoperative radiation therapy or definitive radiation therapy in these patients.[35;36] Women with bulky stage IB or IIA can be treated by chemoradiotherapy, initial surgery followed by chemoradiotherapy or primary radical hysterectomy with complete lymphadenectomy. For the treatment of women with locally advanced (greater than stage IIA) cervical cancer, chemoradiotherapy is preferred. It has been shown that women undergoing radiation therapy and concomitant chemotherapy have up to 50% reduction in the risk of death from cervical cancer compared to radiation therapy alone.[37;38]

The five-year relative survival rates for cervical cancer patients vary from approximately 80% for patients with localized disease to 55% for those with lymph node metastases.[39] Survival rates for patients with more advanced disease at diagnosis are considerably worse.[40;41]

IMMUNE CONTROL OF HPV-INDUCED CERVICAL LESIONS

The life-time risk of a women to ever become infected with one or more HPVs is estimated to be 80%.[42] Yet, the majority of the HPV infections are transient, not even resulting in detectable cervical lesions. Most women infected with a specific HPV type will have cleared the virus in the 6 to 12 months following infection.[43-46] In cases where the immune response fails to clear or control the infection, a persistent infection, often with high levels of high-risk HPV DNA replication, may be established. These persistent infections have an increased probability for clinical progression and thus the development of high-grade CIN lesions and invasive carcinoma.[46-48]

In general, protective immunity results from the interaction of nonspecific innate immunity and antigen-specific adaptive immunity. The first line of defense is the innate immune system, which is activated by cell injury or cell death, so-called danger signals. It is able to clear the majority of pathogens. The innate immune response is aspecific with no memory, but is responsible for activating the adaptive immune response. The adaptive immune response is specific for the antigen(s) involved. Antibody-mediated humoral immunity neutralizes free virus particles and can prevent re-infection. Cell-mediated immune responses, especially cytotoxic T-lymphocytes (CTL), are important for clearance of virus-infected cells and generation of immune memory. Antigen-presenting cells (APC), mainly dendritic cells (DC), appear to be key factors in activating the T-cells, by directing the T-helper cells to either a Th1 or Th2 pathway.[49]

Natural HPV immunity

The exact role which the immune system plays in HPV clearance is unknown. Serum-neutralizing antibody levels are low in natural HPV infections, even the peak titers just after seroconversion.[50] These low levels of antibodies may provide protection against reinfection by the same type. Humoral immunity is generated in most, but not all, infected individuals and is directed against conformational epitope(s) of the major capsid protein L1.[51;52] Recent results with prophylactic vaccines show that vaccinated women are resistant to infection with the HPV types incorporated in the vaccine for at least 48 months post vaccination.[53;54]

While antibody-mediated neutralization of virus plays an important role in preventing infection, cell-mediated immune responses are suggested to be important in controlling established HPV infections as well as HPV-induced (pre) malignant lesions. A longitudinal study of patients who had cleared an HPV infection showed that these patients had strong HPV16 E7-induced T-cell responses around

the time of viral clearance.[55] Scott *et al.* demonstrated that a Th1 cytokine response is associated with subsequent clearance of cervical HPV infection.[56] In addition, strong proliferative cell-mediated immune responses specific for HPV16 E7 peptides are found more often in patients with regression of pre-malignant cervical lesions.[57] Also the correlation between presence of HPV E6-specific CTL and the absence of CIN in women with HPV16 infection indicate that CTL responses may be protective.[58] The importance of cell-mediated immune responses in clearance of established infections has been further indicated by the increased incidence and progression of HPV infections in individuals with immunosuppression, such as transplant recipients or AIDS patients.[59-62] Thus the key factors of a successful immune response to HPV infection, are a strong, local, cell-mediated immunity that is associated with lesion regression and the generation of serum neutralizing antibody, which protects against re-infection.

HPV and immune evasion

Why the immune system fails to detect and clear HPV infection in some cases is not yet fully clear. Various direct and indirect mechanisms used by HPV to evade host immunity have been described. First, the infectious cycle of HPV itself is an immune evasion mechanism. HPV infection does not elicit any danger signals, circumventing the innate immune system. There is no detectable viraemia and the infected cells are not lysed, limiting antigen uptake, delivery to the lymph node, and presentation to naïve B- and T- cells.[63;64] Next, high-risk HPVs down-regulate interferon-responsive gene expression, and the E6 and E7 oncoproteins prevent the immuno-regulatory effects of IFN-α- and IFN-β-antiviral responses.[19;65;66] HPVs minimize the levels of expression of capsid proteins and/or delay expression of these proteins to differentiated epithelium. In this way they avoid Langerhans cells, the APC of the skin. Moreover, it was demonstrated that Langerhans cells are not activated by uptake of HPV capsids. As a result, Langerhans cell migration and maturation, and subsequently the priming of the immune response against the capsid proteins is inhibited.[67]

As a consequence of these immune evasion mechanisms, APC are exposed to low levels of viral proteins in a noninflammatory milieu, leading to local immune nonresponsiveness. HPV antigen-specific effector cells are either not recruited to the infected area, or their activity is down-regulated.[63]

Immune response in patients with CIN and cervical cancer

Low levels of cellular immunity against the HPV E7 oncoprotein can be detected in women with CIN lesions or cervical cancer, as demonstrated in several studies.

Yet, these responses seem unable to clear HPV infections and/or lesions.[68-71] Other studies showed that women with persistent HPV16 infection had no detectable or impaired immune responses against HPV16 E6.[58;72] In contrast, in healthy subjects, frequently an abundant memory Th response against E6 can be observed.[73] These observations suggest that E6-specific CTLs play an important role in protection against persistent HPV infection and associated development of malignancies. Furthermore, it has been shown that in CIN lesions there is a relative down-regulation of TNF-α by the epithelium and up-regulation of the Th2 cytokine IL-10 compared to normal cervix.[74]

Altogether, these observations indeed suggest that patients with CIN lesions or cervical cancer have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens. It is possible that this tolerance arises peripherally at the level of the epithelial keratinocytes, the target cells for HPV. These keratinocytes lack costimulatory molecules such that presentation of viral antigens in the context of MHC class I molecules may result in the induction of anergy in relevant T cells, thus causing immunological tolerance. Steinman and Nussenzweig proposed that peripheral tolerance can also be induced by immature DCs, which silence the T cell repertoire to self and environmental antigens captured in the steady state, that is, in the absence of an acute infection or inflammation. This induction of tolerance by immature DCs would be mediated by peripheral T cell deletion.[75] Immunological tolerance for persistent HPV infection, might develop in a similar manner.

Currently, there is increasing evidence for a possible role of regulatory T cells (Tregs) in the immune evasion mechanisms, contributing to impaired immunity against HPV infection. Tregs form a third subset of CD4+ T cells, beside the two major subsets known as Th2 or Th1 cells, and are characterised by co-expression of CD4, CD25, Foxp3, and lacking CD127 expression (IL-7 receptor). Tregs are thought to recognize self-antigens and function to prevent autoimmunity. However, they also regulate responses to exogenous antigens, and may play a role in controlling chronic and viral infections.[76-78] Increased proportion of Tregs are found in tumor-draining lymph nodes in cervical cancer patients. Lymphocytes in tumor-draining lymph nodes from these patients show a less efficient response to polyclonal activation.[79] TGF-β-producing CD4+/CD25+ T cells were demonstrated in stroma of CIN II/III samples, which suggests that Tregs are recruited to these lesions and may contribute to an immunosuppressive milieu.[80] Visser *et al.* showed that patients with CIN and cervical cancer have increased Treg frequencies in their peripheral blood compared to healthy controls.[81] More importantly, they showed that depletion of CD25+ T cells enhanced the in vitro HPV16 E6 and/or E7-

specific T cell responses in PBMC of half the HPV16-DNA positive cervical cancer patients. These results indicate that Tregs are, at least partially, responsible for the suppression of HPV16 E6/E7-specific T cell responses.

VACCINATION STRATEGIES FOR THE PREVENTION OR THERAPY OF HPV-INDUCED CERVICAL LESIONS

Routine vaccination programs have had an enormous impact on the prevalence of a variety of infectious diseases. The eradication of smallpox [82] and the success of the polio eradication campaign that has reduced the global incidence of this disease impressively, from 350000 polio cases in 1988 to 1948 cases in 2005,[83] illustrate the benefit of vaccination. In light of the fact that HPV is a requirement for essentially every case of cervical cancer and genital warts worldwide, vaccination is likely to be the most effective mechanism to prevent HPV infection and to control HPV-associated disease.

Two different modalities of HPV vaccines are being developed. Firstly, prophylactic vaccines that aim at prevention of an HPV infection are designed primarily to induce virus-neutralizing antibody directed against the capsid proteins of the virus, mainly the L1. Secondly, therapeutic (curative) vaccines that aim at regression of established HPV infections and (pre)malignant cervical lesions. These vaccines are designed to elicit a strong cell-mediated cytotoxic T-lymphocytes response, leading to elimination of cells expressing the oncoproteins of HPV, predominantly E6 and E7. Therapeutic vaccines present far more challenges than prophylactic vaccines. As mentioned earlier, these challenges include the immunocompromised state of cancer patients, difficulty in stimulating the immune system, immune escape mechanisms used by tumors and virally infected cells, and safety issues.

Prophylactic vaccines

An important step forward in the HPV vaccine development came in 1991, when Zhou *et al.* [84] demonstrated that HPV16 L1 capsid proteins expressed in a recombinant system form virus-like particles, so-called VLPs. These VLPs lack the viral DNA, but are morphologically similar to native virus, in that they present all viral conformational epitopes that are highly immunogenic (Figure 5).

Recently, two companies, Merck and GlaxoSmithKline (GSK), have developed commercially HPV VLP vaccines. The Merck vaccine (Gardasil®) is tetravalent. It contains a mixture of four different VLPs of the HPV types 6, 11, 16 and 18. The first two HPV types are considered non-oncogenic but cause approximately 90% of cutaneous genital warts. The latter two types of HPV are found in 70% of cervical

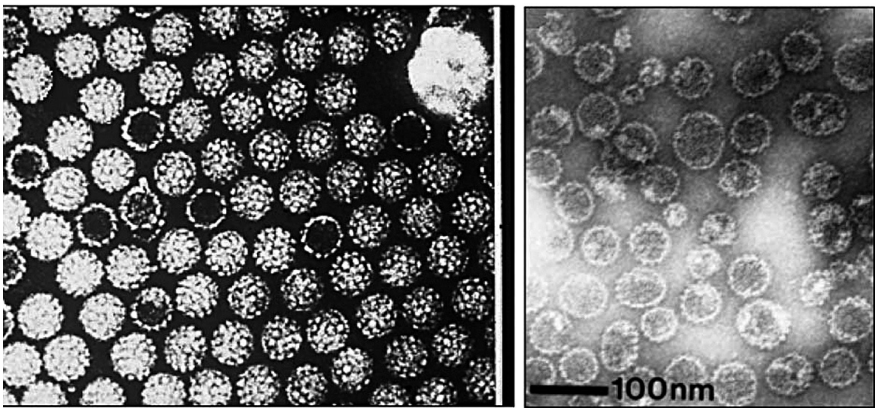

Figure 5. (A) Human papillomavirus particles. (B) HPV-16 L1 virus-like particles made by expressing HPV-16 L1 in baculovirus. (Reproduced, with permission from Elsevier, from Stanley *et al.* Vaccine 2006;24 Suppl 3: S106-S113)

cancer worldwide. Therefore, the Gardasil targets two distinct diseases. The Gardasil VLPs are produced in the yeast *Saccharomyces cerevisiae* and immersed a simple aluminum salt adjuvant. The GSK vaccine (Cervarix®) is bivalent, containing VLPs of HPV16 and HPV18. The L1 protein is produced in recombinant baculovirus-infected insect cells and the vaccine contains the proprietary adjuvant AS04. This adjuvant contains aluminum salts plus monophosphoryl lipid A. Results have been reported of five randomized, placebo-controlled, phase II clinical trials. Both vaccines are given through three intramuscular injections over a six-month period. Both vaccines are well tolerated, not causing vaccine-related serious adverse events. More than 99% seroconversion against each HPV type in the vaccines was induced, and peak antibody titers were at least 50-fold higher than the titers detected after natural infection. In fully vaccinated women, both the vaccines induced full protection from cervical dysplasia associated with the HPV types included in the vaccine and an almost 100% protection against confirmed persistent infections by the same types.[50;53;54;85;86] As of November 2006, the Gardasil® vaccine from Merck is available in the Netherlands. Subsequently, in 2007, Cervarix® is approved in Australia and Europe. Two of the large Phase III trials of the tetravalent vaccine and one of the bivalent vaccine have been completed, and other Phase III trials are still underway.[87-90] Although these highly effective and apparently safe vaccines have great potential for reducing cervical cancer rates and the number of surgical treatments for pre-malignant cervical lesions, several unresolved issues remain. Only limited follow-up in the Phase II and III trials has been undertaken. Therefore, the duration of protection after vaccination is unknown. Given that vaccination will not protect against the HPV

types not included in the vaccines, around one third of the cervical cancers will still develop despite vaccination. Screening will therefore need to be continued. Besides, it will take decades before the impact of the vaccine on cervical cancer is observed, as the target population for vaccination will be 9-13-year old females. Some "catch-up" vaccination of older, sexually-active women will occur in many countries. However, this will presumably involve much lower rates of coverage compared to cohort vaccination of young adolescents. It is likely that the current approach of frequent screening will prove to be too expensive and inefficient for many countries. Education of physicians, government and general public will be essential for a successful implementation of the prophylactic HPV vaccines. Finally, the high cost of the first generation HPV vaccines will be a major barrier for their introduction in the world's poorest countries, where the vaccines are needed most. To achieve HPV vaccination in developing countries, it will be essential that vaccine manufacturers, governments and non-governmental organizations work together to provide vaccination at an affordable cost.[91;92]

Therapeutic vaccines

As explained earlier, even if prophylactic vaccination were introduced on a worldwide scale today, it will take decades before the incidences of HPV-induced premalignant lesions and invasive cervical cancer will decrease. Therapeutic vaccines are needed to fill in this niche, by attacking already established HPV infections and HPV-induced disease.

Whereas most tumor-specific antigens are derived form normal or mutated "self" proteins, E6 and E7 are completely foreign, viral, proteins, and thus they harbor more antigenic peptides/epitopes than a mutant "self" protein. Furthermore, the expression of E6 and E7 occurs in cells in which the viral genome has integrated into the cellular DNA, and is necessary for the virus-infected cell to escape apoptosis and cell-cycle arrest. Therefore, constitutive production of these oncoproteins is required for the maintenance of the transformed phenotype of (pre)malignant cells. As a consequence, cervical cancer cells are unlikely to evade an immune response through antigen loss. Thus, E6 and E7 represent attractive targets for developing immunotherapies or vaccines against cervical cancer.

There are substantial numbers of publications describing preclinical model studies of therapeutic vaccination strategies and several candidate vaccines made it into Phases I and II clinical trials. Below, a brief overview is given of the various forms of HPV vaccines that have been evaluated clinically.

Peptide vaccines.

Peptide vaccines have the advantages of safety, ease of production, and cost

effectiveness. However, these vaccines are weakly immunogenic and require HLA compatibility. It is possible to predict immunodominant or subdominant peptides of viral antigens that would associate with a particular HLA allelic product and that are recognized by human T cells. Since 40% of Caucasians carry the HLA-A2 allele, HPV16 E7 peptides presented by this allele have been the antigen in several phase I/II clinical trials. Upon peptide vaccination no adverse side-effects were observed, Th responses were induced, enhancement in cytokine release and CTL activity could be measured in a majority of the patients. Yet, less than 20% of the patients had partial clearance of virus and regression of lesions, and the studies were performed on a limited number of patients (<20).[93-96]

Zwaveling *et al.*[97] showed that vaccination with longer peptides resulted in more potent CTL responses than vaccination with exact minimal CTL epitope length. By increasing the size of the peptide, it is forced to be presented by professional APC, like protein vaccines, which enhances the vaccination efficacy. A clinical trial is underway in patients with HPV16 associated neoplasia.

Protein vaccines.

The advantage of recombinant proteins over peptide approaches is that they deliver all potential epitopes to the APC of the immune system. Since these APC process and present one or more peptide epitopes in association with host HLA molecules, these vaccines can be used regardless of the individual's tissue type. In addition, protein vaccines offer certain safety advantages as potential concern with certain recombinant virus vaccines and DNA vaccines related to integration of genetic material into the host genome and cell transformation are not an issue.

A fusion protein consisting of HPV16 L2 fused to E7 protein (TA-GW), has been tested for clinical treatment of genital warts. Vaccination with TA-GW appeared safe, well-tolerated and immunogenic.[98;99] Immunization with another fusion protein, which consists of HPV16 L2/E6/E7 (TA-CIN) resulted in E7-specific CD8+ T-cell immune responses and tumor protection in mice.[100] Immunization with TA-CIN fusion protein was well-tolerated by patients and induced both humoral and T-cell mediated immune responses.[101] Phase I/II trials have been conducted with another fusion protein, containing a mutated HPV16 E7 linked to the first 108 amino acids of *Haemophilus influenzae* protein D. Vaccination led to limited regression of lesions in 3 of the 5 patients with CIN III, it was well tolerated and led to significant CD4 and CD8 T-cell lymphocytic infiltration.[102]

The potency of protein vaccines may be further enhanced through the use of adjuvants or fusion with heat-shock proteins. It was demonstrated that a vaccine containing heat-shock protein and E7 protein protected mice against challenge and rechallenge with an E7-expressing murine tumor cell line.[103]

Reconstituted viral envelopes (virosomes) appear to be ideally suited for delivery of protein antigens to the cytosol of APC, and thus for introduction of antigenic peptides into the MHC class I presentation pathway.[104;105] Virosome-mediated delivery of protein antigens circumvent problems associated with MHC restriction and HLA polymorphism in the human population, since the APC would select its own peptides. Recently, Bungener *et al.* in our laboratory showed that upon immunization with virosome-encapsulated HPV16 E7 protein, induced strong CTL responses, and tumor protection could be induced in a murine model system.[106] Chapter 8 of this thesis presents a study in which E7-containing virosomes are used in conjunction with recombinant SFV in a heterologous prime-boost immunization strategy.

Chimeric HPV VLP vaccines represent innovative protein-based HPV vaccines. Generally, in these vaccines, a fragmented E7 protein is attached to the L1 VLP. It has been shown that VLPs can induce high-titer neutralizing antibodies, activate DCs, and prime T-cell mediated immune responses.[107-111] Several of these vaccine candidates are in the early stages of clinical evaluation.[10]

DNA vaccines.

DNA vaccines are useful because of their purity, ease of preparation and stability. Immunization with DNA vaccines results in extended expression of antigen on MHC-peptide complexes over a longer period of time compared with peptide or protein vaccines. By directly transducing DNA coding for antigen into APC, proteins are synthesized and antigenic peptides presented by the patient's own HLA molecules. DNA vaccines can be administered by intramuscular injection, intradermal injection via hypodermic needle or gene gun, intravenous injection, intranasal delivery or biojector delivery.[112;113]

Since naked DNA vaccines are weakly immunogenic, various strategies have been developed to enhance their immunogenicity. Co-administration of E7-DNA with DNA encoding anti-apoptotic proteins has been demonstrated to enhance, E7-specific immune responses and anti-tumor effects.[114] Another strategy to improve the antigenicity of HPV DNA vaccines involves encapsulation of the DNA in the delivery system. Garcia *et al.*[115] reported on the use of encapsulated plasmid DNA-encoding fragments derived form E6 and E7 of HPV16 and HPV18 in biodegradable particles (ZYC101a). In a randomized double-blind controlled trial they showed significantly higher rates of CIN II/III resolution in the treated groups under the age of 25 years. However, there was no difference in resolution rates between vaccine and placebo in the group older than 25 years of age. Another way to enhance the potency of DNA vaccines is through linking E7 to HSV-1 VP22 or one of its homologues, thereby facilitating the spreading of the E7. In mice vaccination

with VP22/E7 DNA resulted in significantly higher number of E7-specific precursor CD8+ T-cells and a stronger antitumor effect compared to wild-type E7 DNA[116-118]

***DC-based vaccines**.*

*D*C-based vaccines are highly immunogenic. On the other hand, labor-intensive individualized cell processing is required. In three patients with cervical cancer it was demonstrated that recombinant, full-length, E7-pulsed, autologous DCs could induce both E7-specific CD4+ T-cell responses and strong CD8+ CTL responses capable of lysing autologous HPV-infected cancer cells.[119] A Phase II trial of an HPV16 and 18 E7-pulsed DC-based vaccine demonstrated that it induced E7-specific humoral and cell-mediated immunity. Yet this response did not lead to a clinical response.[120] In another trial, HPV E7 antigen-loaded autologous DCs were evaluated in stage IV cervical cancer patients. Again, though vaccination induced T-cell responses in some patients, no clinical response was observed.[121]

***Tumor cell-based vaccines**.*

Tumor cell-based vaccines represent genetically modified tumor cells encoding co-stimulatory molecules or cytokines that may enhance immunogenicity, which may lead to T-cell activation and antitumor effect after vaccination.[122] Several preclinical studies showed that vaccines using HPV-transformed tumor cells transduced with cytokine genes, such as interleukin-12, interleukin-2, or GM-CSF, are able to induce strong antitumor effects.[123;124]

***Bacterial vector vaccines**.*

Bacterial vector vaccines have the advantage of being highly immunogenic and are able to deliver plasmids or express proteins. Drawbacks are the potential safety concerns, and possible pre-existing immunity limiting their clinical application. Different attenuated bacteria (e.g., *Listeria monocytogenes, Salmonella, Shigella, Escherichia Coli*, *Mycobacterium bovis*) can be used as bacterial vectors to deliver either plasmids encoding genes of interest or proteins of interest to APC.

Gunn *et al.*[125] was the first to demonstrate that vaccination with recombinant *L. monocytogenes* secreting HPV16 E7 can lead to regression of pre-existing E7-expressing tumors using an E7-expressing murine tumor model. Orally administered antigen-specific *L. monocytogenes* vaccines may also induce potent immune responses and antitumor effects in murine models.[126]

***Viral vector vaccines**.*

Viral vector vaccines have the advantage of being highly immunogenic, as they express the different immunogenic properties of viruses which the vectors are derived from. Drawbacks include potential toxicity and pre-existing viral immunity in the recipient.

Several preclinical studies demonstrated that immunotherapy using vaccinia

vectors generated strong CTL activity and antitumor responses against E6 and/or E7. Phase I and II clinical trials using recombinant vaccinia virus encoding HPV16 and 18 E6/E7 (also called TA-HPV) established the safety and indicated that some patients with CIN lesions or advanced cervical cancer developed T-cell immune responses upon vaccination.[127-129] TA-HPV has also been used in the treatment of high grade HPV16-positive vulval intraepithelial neoplasia (VIN). Most patients who received a single dose of the vaccine demonstrated HPV-16-specific immune responses. However, no complete correlation between immunological and clinical responses could be defined.[130]

A recombinant viral vector that has been evaluated extensively in human clinical studies for treatment of a variety of illnesses is the adenovirus system. Adenoviral vectors have a cloning capacity of approximately 8 kb, allowing for insertion of relatively large genes. They can be prepared easily in high titer and efficiently transduce a wide range of cell types. Concerns were raised about the use of adenovirus vectors after a report describing a fatal systemic inflammatory response in a patient following adenoviral gene transfer.[131;132] Another major concern for immunization is the presence or production of anti-adenoviral antibodies, which may hamper repeated vaccinations and thereby may compromise the therapeutic effect. Several studies using modified adenovirus-expressing HPV16 E6 and/or E7 showed enhanced antigen-specific CD8+ and/or CD4+ T-cell immune responses induced upon immunization in mice.[133;134] In Chapter 7 of this thesis the therapeutic efficacy of a recombinant adenoviral vector expressing HPV16 E6E7 is compared to that of recombinant SFV in a a murine model system. Another application of adenovirus is an adenoviral vector encoding E7 and targeted to CD40 by means of bispecific antibodies. DCs infected by this adenoviral vector enhanced protection against HPV16-induced tumor cells in a murine model, and could initiate partial therapeutic immunity in mice bearing established tumors. This protection was both antigen-specific and CD8+ T-cell dependent.[135] Given that vaccinia and adenoviruses are DNA viruses, another potential concern of using these vectors to deliver HPV E6 and E7 oncogenes in therapeutic vaccines is the integration of these oncogenes into the host genome.

Vectors based on alphaviruses (i.e. Sindbis virus, Semliki forest virus, and Venezuelan equine encephalitis virus) are attractive candidates for vaccine development and are gaining increasing interest for their superiority over other viral vectors with respect to the induction of cellular and humoral immune responses. Alphavirus vectors are recombinant RNA viruses with a self-replicating RNA genome. Hence, there is no concern for integration of the transgene into the host cell chromosome. Furthermore, the majority of individuals have no

pre-existing immunity to these viruses.[136] Hence therapeutic alphavirus-based vaccination strategies are very promising and are likely to be taken into clinical trials in the near future. This thesis focuses on the immunotherapeutic effect of a recombinant SFV viral vector encoding a fusion protein of HPV16 E6 and E7 in a murine model system. A detailed overview of alphaviral vectors is given in Chapter 2 of this thesis.

Heterologous prime-boost strategies

Heterologous prime-boost strategies involve priming the immune system to a target antigen delivered by one vector and then selectively boosting this immunity by re-administration of the antigen in the context of a second distinct vector. With this strategy powerful synergistic effects can be achieved, reflected in an increased number of antigen-specific T cells, selectively enrichment of high avidity T cells and increased efficacy against pathogen challenge.[137;138] Additionally, these protocols may generate improved effector memory CD8+ T cell responses.[139]

Chen *et al.* showed that priming with a DNA vaccine followed by a recombinant vaccinia booster enhanced E7-specific $CD8^+$ T cell precursor frequencies.[140] A heterologous prime-boost clinical study with TA-CIN with TA-HPV demonstrated enhanced immunogenicity compared with either agent alone. The order of TA-CIN followed by TA-HPV was superior, inducing the highest number of T cells against the oncoproteins.[100] In patients with anogenital intraepithelial neoplasia this prime-boost regime induced both humoral and cellular immunity, yet no simple relationship between induction of systemic HPV16-specific immunity and clinical outcome could be obtained.[141]

SCOPE OF THIS THESIS

Aim

The aim of the study described in this thesis was to develop an immunotherapeutic strategy against CIN lesions and cervical cancer. Persistent high-risk HPV infection with continued expression of both oncoproteins, E6 and E7 are a prerequisite for the development of invasive cervical lesions. Patients with CIN lesions or cervical cancer might be immuno-suppressed and/or have mounted a certain degree of immunological tolerance or ignorance for these HPV-derived oncoproteins. This puts high demands on potential immunotherapeutic strategies, since such approaches need to overcome this tolerance in order to be effective. Viral vectors are being developed for immunotherapy of cancer and infectious diseases. As indicated above, vector based on alphaviruses are gaining increasing interest for

their superiority over other viral vectors with respect to the induction of cellular and humoral immune responses. This thesis mainly focuses on the use of a therapeutic immunization strategy against HPV-induced cervical cancer based on such an alphavirus vector, i.e. Semliki Forest virus.

Summary of the Chapters

In **Chapter 2**, an overview of recombinant viral vectors based on alphaviruses is given. After a brief introduction on alphaviruses, describing their structure, life-cycle and replication, the development and design of recombinant vector systems based on alphaviruses are described. In addition, an overview is given of current preclinical immunotherapy studies using these vector systems. Chapter 2 also presents a summary of the immunotherapeutic effects of recombinant SFV in our murine HPV model system, as detailed in the following chapter of this thesis.

In **Chapter 3**, the efficacy of SFV-enhE6,7 is investigated in a mouse model. The therapeutic effect of immunization was examined by inoculating mice at different time points with tumor cells prior to immunization. The memory immune response induced upon immunization was determined by re-challenge three months after the initial challenge and immunization. Also, CTL responses in mice were studied up to 340 days after immunization and tumor challenge.

In **Chapter 4**, the effects of the route of immunization and dose administered of SFV-enhE6,7 are investigated. First, the induction of precursor CTL frequencies and bulk CTL activity upon either intramuscular (i.m.), intravenously (i.v.), subcutaneously (s.c.) or intraperitoneally (i.p.) immunization with SFV-enhE6,7 were determined. Subsequently, the minimal effective dose of s.c. or i.v. SFV-enhE6,7 were investigated using a standard bulk CTL assay. Next, the therapeutic efficacy of i.m., i.v., and s.c. SFV-enhE6,7 immunization were compared in a tumor treatment experiment. The efficacy of i.m. and i.v. immunization of SFV-enhE6,7 was further explored by initiating immunization at later time points after the tumor challenge or by lowering the dose of SFV-enhE6,7.

In **Chapter 5**, the potency of SFV-enhE6,7 is further explored in an immune-tolerant K10 HPV16-E6/E7 transgenic mouse model. These transgenic mice constitutively express HPV16 E6 and E7 under the control of the keratin 10 promoter in the suprabasal layers of the epidermis. E7-specific CTL tolerance in these mice is strong, as it can not be broken by immunizations using naked DNA or E7 protein mixed with an adjuvant. The induction of E6/E7-specific CTL activity upon immunization with SFV-enhE6,7 was determined by bulk CTL assay and INF-γ Elispot analysis in these mice. Also the effect of the route of administration (i.e. s.c., i.m., and i.v.) on the induction of CTL activity in these mice was investigated.

Furthermore, the efficacy of immunization with SFV-enhE6,7 was compared to that of VP22-$E7_{1-60}$ DNA.

Chapter 6 describes a study which evaluates whether systemic addition of SFV-IL12 to immunization with SFV-enhE6,7 improves the induction of antigen specific CTL activity and anti-tumor response. Treatment with IL12 has shown to have a marked immune-activating and anti-tumor activity. Also the use of IL12 as an adjuvant resulted in potentiated antigen-specific CTL responses and increased anti-tumor therapeutic efficacy. Furthermore, co-administration of tumor antigens and IL12 has been described to provide an environment with inflammatory danger signals that is required to activate DCs and may thereby prevent or revert tolerance to tumor-associated antigens. First, the most optimal amount, route, and timing of immunization of SFV-IL12 with SFV-enhE6,7 was determined. Next, this optimal immunization regiment was evaluated in K10 HPV16-E6/E7 transgenic mice. Finally, the therapeutic efficacy to eradicate established tumors was analyzed in a tumor-treatment experiment.

In **Chapter 7**, as a prelude to future clinical evaluation of SFV vector, the efficacy of rSFV with a recombinant adenoviral vector is compared, since adenoviral vectors have been and are being used in numerous clinical trials. Differences were investigated with respect to CTL induction and anti-tumor response in a murine HPV-tumor model. Additionally, to unravel the observed differences between the vectors, T-cell depletion and gene expression experiments were conducted.

In **Chapter 8**, a heterologous prime-boost strategy with SFV-enhE6,7 and E7-virosomes is described. Such strategies are found to establish higher frequencies of antigen-specific T cells than homologous prime-boost protocols or single immunizations. The efficacy of this protocol was analyzed by determining the induction of antigen-specific precursor CTL activity, bulk CTL activity and anti-tumor activity compared to homologous prime-boost protocols. Furthermore, it was investigated whether the potency of a heterologous booster immunization with SFV-enhE6,7 is affected by vector-specific immunity induced during the prime immunization, by mixing irrelevant rSFV with E7-virosomes during the priming immunization followed by a SFV-enhE6,7 boost.

Chapter 9 presents a general discussion of the results described in the thesis. Also, it provides a perspective on the opportunities and challenges related to the introduction of therapeutic HPV vaccination, particularly therapeutic vaccination based on the use of the recombinant SFV vector system, against the background of the ongoing implementation of prophylactic HPV vaccination in many countries.

In **Chapter 10**, a summary of this thesis is given.

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CHAPTER 2

Recombinant alphaviruses as vectors for anti-tumour and anti-microbial immunotherapy

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J Clin Virol. 2006 Mar;35(3):233-43

ABSTRACT

Background: Vectors derived from alphaviruses are gaining interest for their high transfection potency and strong immunogenicity.
Objectives: After a brief introduction on alphaviruses and their vectors, an overview is given on current preclinical immunotherapy studies using vector systems based on alphaviruses. The efficacy of alphavirus vectors in inducing immune responses will be illustrated by a more detailed description of immunization studies using recombinant Semliki Forest virus for the treatment of human papillomavirus-induced cervical cancer.
Results: Immunization with recombinant alphavirus results in the induction of humoral and cellular immune responses against microbes, infected cells and cancer cells. Preclinical studies demonstrate that infectious diseases and cancer can be treated prophylactically as well as therapeutically.
Conclusions: Alphavirus-based genetic immunization strategies are highly effective in animal model systems, comparing quite favourably with any other approach. Therefore, we hope and expect to see an efficient induction of tumour- or microbial immunity and a positive outcome in future clinical efficacy studies.

INTRODUCTION

Vectors based on alphaviruses are gaining increasing interest for their superiority over other viral vectors with respect to the induction of cellular and humoral immune responses. Currently, prophylactic and therapeutic vaccines for infectious diseases and cancer based on these vectors are being developed. The prototypic vectors are derived from Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus. Recombinant alphavirus particles carry the RNA that code for the replicase and the heterologous gene but lack the RNA that codes for the structural proteins. Consequently, infection of cells with recombinant alphavirus vectors will not result in productive replication and the virus will not spread beyond initially infected cells.

Before giving an overview of the current (pre)clinical studies using alphavirus vectors we will briefly introduce alphaviruses and their derived vector systems. We will illustrate the high efficacy of alphavirus vectors in inducing immune responses by a more detailed description of our immunization studies using recombinant Semliki Forest virus for the treatment of human papillomavirus-induced cervical cancer.

Alphaviruses

Alphaviruses are small, enveloped, positive-strand RNA viruses belonging to the family *Togaviridae*. The alphavirus genus comprises 27 different members including Semliki Forest virus (SFV), Venezuelan Equine Encephalitis virus (VEE)

and Sindbis virus (SIN), from which vector systems have been developed. SFV and SIN were originally isolated from mosquitoes. SFV is named after the Semliki Forest (Uganda) and SIN after the Egyptian village Sindbis[1]. VEE was first recognized as the causative agent of infectious equine encephalomyelitis in Venezuela.

Alphaviruses are naturally transmitted by mosquitoes to vertebrates, and in turn back to mosquitoes[1]. In vertebrate cells, virus infection results in the rapid shutoff of host mRNA translation, take over of the translational machinery by viral mRNAs, production of high titres of infectious virus and eventually cell death by apoptosis. In mosquito cells virus replication is slower and often has minimal effects on the cell. Natural vertebrate hosts are avian and mammalian species. Although there is a risk of infection, alphaviruses are not major pathogens to humans. The spectrum of alphavirus disease in humans ranges from silent asymptomatic infections or undifferentiated febrile illness (SFV) and mild polyarthritis (SIN) to encephalitis (VEE). The incidence in humans is very low. Nonetheless, especially for vectors derived from VEE, biosafety features in the vector system are essential to prevent formation of infectious virus. Yet, also for vector systems derived from SFV and SIN these biosafety aspects are incorporated, as will be explained below.

Alphavirus structure

As the structure of SIN and SFV has been studied in considerable detail, the information in this and the following paragraph is based on these two type-specific members of the alphaviruses[2]. Alphaviruses are spherical particles with a diameter of 65-70 nm. The viral genome consists of a single stranded RNA genome surrounded by a capsid, together forming the nucleocapsid. The capsid is formed by a regularly arranged icosahedral (20-faced) shell composed of 240 copies of one protein: the capsid protein. The nucleocapsid is enveloped by a lipid bilayer derived from the host-cell plasma membrane into which 240 copies of the glycoproteins, E1, E2 for SIN and E1, E2 and E3 for SFV, are inserted. The glycoproteins form 80 hetero-oligomeric spikes. Each spike consists of a trimer of E1/E2 heterodimers (SIN) or E1/E2/E3 heterotrimers (SFV)[3]. These polypeptide chains span the lipid bilayer and interact with the C protein. The spike-forming trimers projecting outward from the surface of the virus can be seen by electron microscopy (Figure 1). The alphavirus genome consists of a single RNA molecule of positive polarity which is capped and polyadenylated and serves directly as an mRNA once introduced in the host cell. It consists of two open reading frames (ORF). The first ORF codes for the four non-structural proteins of the virus that form the replicase complex (nsP1-4). The second ORF encodes the structural proteins of the virus: the capsid (C) protein and the envelope proteins E2 (synthesized as

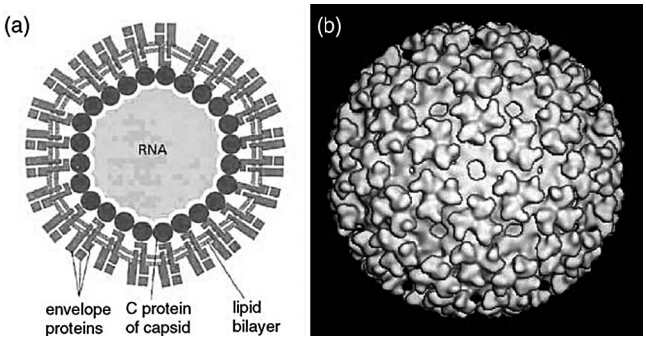

Figure 1. The SFV structure. The nucleocapsid (RNA and capsid together) is surrounded by a lipid bilayer membrane envelope into which viral spikes projecting outward from the surface of the virus are inserted (a). The viral spikes can be visualized in cryo-electronmicroscopy reconstruction images (b) (Panel A with permission from: Alberts B, Bray D Lewis J, Raff M, Roberts K, Watson JD: Molecular Biology of the Cell, third edition, 1994, Garland Publishing, pp. 274–279; Panel B by courtesy of S. Mukhopadhyay and J. Smit).

a larger precursor P62 (SFV) or PE2 (for SIN)), 6K and E1, as shown in Figure 2.

Alphavirus life-cycle and replication

Alphaviral infection is initiated by binding of the viral envelope protein to a cell surface protein that serves as its receptor on the host-cell plasma membrane[2]. For SFV several proteins have been suggested as functional receptors and consistent with its broad host range, SFV is probably able to utilize a variety of surface receptors with varying affinity. After binding, the virus enters the cell by clathrin-mediated endocytosis and is transported to endosomes. The acidic pH within the endosomal compartment causes the viral spikes to mediate fusion between the viral and the endosomal membranes[4;5]. The nucleocapsid then is released into the cytosol, where uncoating of the nucleocapsid by ribosomes is followed by release of the viral RNA into the cytoplasm. After fusion with the endosomal membrane the viral glycoproteins are transported to lysosomes to be degraded[6]. Recently, the regulation of this intracellular transport mechanism was further characterized demonstrating that Rab7 (a small GTPase) is recruited to early endosomes, where

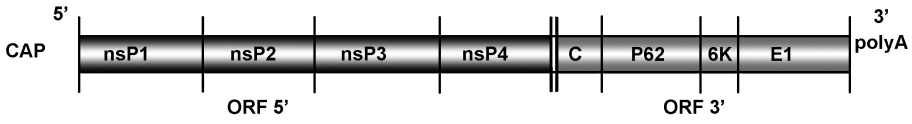

Figure 2. The wild-type SFV genome consists of two open reading frames; the first of which encodes the four non-structural proteins that form the replicase complex, the second codes for the structural proteins.

it forms distinct domains that mediate cargo sorting as well as the formation of late-endosome-targeted transport vesicles[7].

Alphavirus infection results in the shutoff of host proteins synthesis in favour of viral translation. Recently, McInerney *et al.*,[8] demonstrated that for SFV the inhibition of host protein synthesis is due to the activation of the cellular stress response resulting in the formation of stress granules[9]. Stress granules (SG) are cytoplasmic domains into which mRNAs are sorted in response to phosphorylation of eukaryotic initiation factor eIF2-alpha, a key regulatory step in translation initiation. This mechanism enables stressed cells to shut down the expression of normal house-keeping genes to allow the selective expression of stress response factors. The mRNA is believed to be stored, pending either degradation or resumption of normal translation in the absence of stress. During SFV infection, SG formation is transient and occurs at the time of host shutoff, i.e., when the profile of protein production changes from cellular to viral[8]. SFV-induced SGs dissolve in the vicinity of viral RNA as replication progresses.

Viral replication begins with translation of the first ORF, which codes for the four non-structural proteins nsP1, nsP2, nsP3 and nsP4 that make up the viral replicase. This replicase initially catalyses the formation of a full-length negative strand intermediate – the 42S RNA – from which more genomic RNA is produced. These genomic positive-strand RNA molecules are capped by the replicase at the 5'-end. Secondly, the replicase catalyses transcription of the second ORF to form a subgenomic 26S RNA molecule that codes for the structural proteins. This subgenomic RNA is replicated to large amounts, leading to a high production of the structural proteins. The newly synthesized capsid and envelope proteins follow separate pathways through the cytoplasm. The capsid protein, like the cytosolic proteins of the cell, is synthesized by ribosomes that are not membrane-bound. The newly synthesized genomic RNA and capsid protein are rapidly associated into new nucleocapsids in the cytosol. In contrast, the envelope proteins, like the plasma membrane proteins of the host cell, are synthesized by ribosomes that are bound to the ER. Rapidly after their synthesis P62 and E1 (SFV) or PE2 and E1 (SIN) form heterodimers. These future envelope proteins are inserted into the membrane of the ER, where they are glycosylated, transported to the Golgi apparatus, and then delivered to the plasma membrane. At a compartment after the trans-Golgi network, but prior to appearance at the cell surface, the P62 or PE2 precursor is cleaved into the mature moieties E2 and E3. The viral nucleocapsids and envelope proteins finally meet at the plasma membrane. As a result of an interaction between the C proteins in the nucleocapsid and the cytoplasmic tail of E2, the nucleocapsid forms a bud whose envelope contains the envelope proteins

embedded in host-cell lipids. Finally, the bud pinches off and a free virus particle is released on the outside of the cell. The clustering of envelope proteins as they assemble around the nucleocapsid during viral budding excludes the host plasma membrane proteins from the final virus particle.

Recombinant vector system based on alphaviruses

Alphavirus-based expression vectors have been constructed according to two fundamental designs. On the one hand replicon vectors, generating recombinant replicon particles that are limited to one round of infection and on the other hand double-genomic vectors that generate replicating recombinant virus. In both designs the recombinant RNA is self-replicating and expresses the foreign gene(s) at high levels. Yet, in the replicon system transgene expression is transient while in the replicating system transgene expression is more sustained. Next to replicon particles, alphavirus vaccines are being developed consisting of naked DNA/RNA replicons.

Replicon vectors

Using SIN, Xiong *et al.*[10] were the first to develop a replicon expression vector based on an alphavirus. Later on replicon vectors were developed using SFV and VEE virus[11;12]. The principles of the SIN and VEE replicon systems are in essence similar to the vector system based on SFV, which is described below.

Liljeström and Garoff[11] developed the SFV-based replicon vector system. The full-length cDNA copy of the viral genome was cloned in a bacterial plasmid including a prokaryotic DNA-dependent RNA polymerase such that viral RNA can be transcribed *in vitro*. These RNA transcripts are fully infectious, i.e. introduction into cells suffices to initiate replication and a full infection cycle, resulting in virus formation. Next the alphavirus RNA replication and packaging machinery was adapted for expression of heterologous RNAs and proteins in animal cells. The structural proteins of SFV have been deleted and replaced with a polylinker into which foreign genes can be inserted (Figure 3). As the RNA is self-amplified by the replicase complex, high level expression of the foreign gene is obtained. The helper vector(s) codes for the capsid and spike proteins (Figure 4). Recombinant SFV (rSFV) virus-like particles can be generated by cotransfection of cells with the recombinant RNA vector and a helper RNA vector (Figure 5). The RNA packaging signal is located in the non-structural region of the vector and absent on the helper vector. Thus, only the recombinant RNA is packaged into newly generated virus particles that are released from the packaging cell. This helper system provides a first line of biosafety in that virus particles are formed that lack the genes encoding the

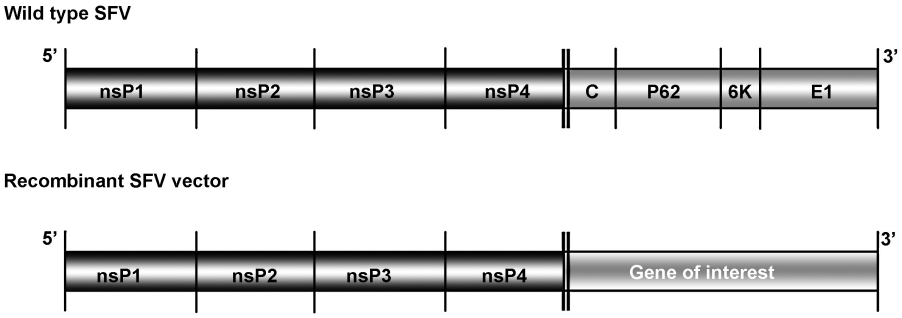

Figure 3. Schematic presentation of the wild-type SFV genome and the recombinant SFV vector. For use of alphaviruses as recombinant vector systems, the structural proteins are deleted and a gene of interest can be inserted.

structural proteins. For use in humans, increased biosafety can be obtained by the split helper system (Figure 4)[13]. Splitting the helper plasmid in two helper plasmids decreases the probability of formation of infectious, replication competent virus, as recombination between the two helper and the vector plasmids is highly unlikely. Since the RNA encoding the structural proteins is not encapsidated, the recombinant particles that are generated undergo only one round of infection, being unable to produce progeny virus. These rSFV particles are therefore also termed "suicide" particles. Upon infection, the recombinant RNA is expressed to high levels, including the gene of interest that is inserted into the multiple cloning site. Expression is transient as infected cells undergo apoptotic cell death.

More recently it was shown that by the introduction of a translational enhancer

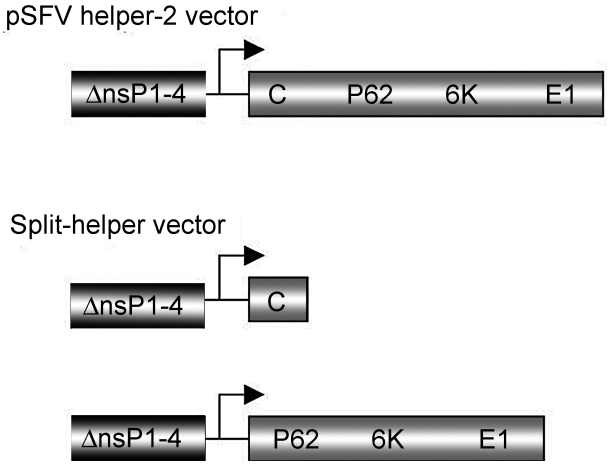

Figure 4. Schematic presentation of pSFV helper constructs (see text).

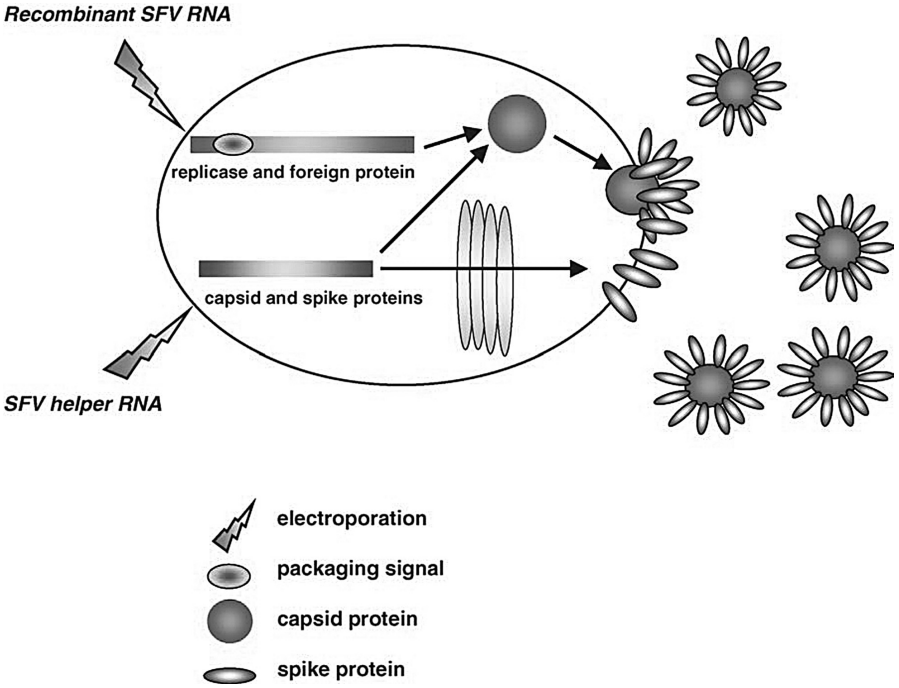

Figure 5. Schematic representation of the production of recombinant SFV. In vitro, cells are electroporated in the presence of recombinant SFV RNA and helper RNA(s). Upon introduction of these RNAs into the cytoplasm of cells the RNAs are replicated and translated as described in the text. Subsequently, the viral recombinant RNA (which contains a packaging signal) associate with the capsid protein. The RNA-capsid complex assembles at the cellular membrane with spike proteins forming recombinant virus particles that are released by the cell.

element in the SFV vector system the expression of the foreign gene can be strongly enhanced. This enhanced vector contains the first 34 amino acids of the SFV capsid gene and the foot and mouth disease virus (FMDV) 2A protease downstream of the 26S promoter. Foreign genes are cloned in frame with this translational enhancer element, which results in enhanced translation of the gene. The FMDV protease is included so that the enhancer element is cotranslationally removed from the foreign protein. Expression levels in cells transfected with these "enhanced" rSFV particles are up to 10-fold higher than those transfected with the standard rSFV particles[14]. Although the mechanism of the enhancement is not entirely clear it was demonstrated that translation is only enhanced in the presence of intact eIF2-alpha (translation initiation factor) phosphorylation; these cells form stress granules upon SFV infection (see above). The authors suggest that the SFV translational enhancer counteracts the translation inhibition imposed by eIF2-alpha posphorylation[8].

Replicating vector systems

Next to these suicide replicon vectors, replicating vectors based on alphaviruses are being developed that allow more prolonged gene expression. In the first type of replicating vectors the transgene is placed under the transcriptional control of a duplicate 26S promoter inserted within the 3' nontranslated region of the viral genome or within the short nontranslated region located just upstream of the native 26S promoter[15]. Since double-subgenomic vectors retain all viral genes, they are capable of multiple rounds of infection and result in a more sustained transgene expression. Yet, eventually also with these vectors gene expression is transient as, similar to the replicon particles, cells infected with these replicating virus particles die and the virus is cleared by the immune system. As replicating vectors may be of great value for future (immuno)therapy of cancer or infectious diseases several other strategies are being investigated to generate replicating alphavirus vectors[16-19]. Yet, as the number of studies using replicating virus is limited so far, we will, in the next paragraphs, focus on the use and applications of replication-defective replicon particles.

Immunization strategies based on alphavirus vectors

Prophylactic vaccination against infectious diseases in general aims at the induction of humoral immune responses to prevent infection. This humoral immune response is mediated by plasma cells, i.e. antibody-producing B cells. On the other hand, therapeutic immunisation against infected cells and tumour cells requires the induction of cytotoxic T lymphocytes (CTL) that can specifically recognise and lyse infected cells or tumour cells. For the differentiation, expansion and memory induction of plasma cells and tumour- or microbe-specific CTLs, T helper cells (Th cells) are required. And, as key orchestrators in these processes of both humoral and cellular immune responses, properly activated antigen-presenting cells, dendritic cells (DCs) in particular, are essential.

Characteristics of alphavirus-based immunization strategies

Vectors based on alphaviruses are gaining increasing interest because of their superiority over other viral vectors with respect to the induction of both humoral and cellular immune responses. This superiority can be ascribed to several features of alphavirus-based immunization strategies but presumably lies in the combined effects of these features. Characteristics that make alphaviruses attractive candidates for development of replication-defective viral vectors for application in humans are that (i) recombinant alphaviruses induce high-level expression of encoded proteins, (ii) after 48-72 h of protein expression infected cells die by

apoptosis resulting in apoptotic bodies containing high levels of the transgene protein which may be very beneficial for the induction of immune responses via cross-priming[20], (iii) recombinant alphaviruses activate both the innate and the adaptive immune system. Infection of cells results in dsRNA intermediates that are known for their immunopotentiating capacity[21]. dsRNAs can be recognized by innate immune receptors such as Toll-like receptor 3 and trigger production of Interferon type I, while, in addition, dsRNAs activate and induce maturation of DCs[22], (iv) humans in general do not carry neutralizing antibodies against SIN or SFV that may decrease the efficacy of the immunization. In addition, Berglund *et al.*[23] demonstrated that upon immunization of mice with rSFV the immune responses against the SFV vector itself did not disable boost responses by subsequent immunizations with the same vector.

With respect to the delivery of encoded protein antigen to DCs for MHC class I and MHC class II processing and presentation one can envision two alternative mechanisms: (i) recombinant alphaviruses transfect DCs directly thereby inducing synthesis of the encoded antigen in the cytosol followed by MHC processing and presentation (direct priming) or (ii) the recombinant virus particles transfect other cell populations. When these cells, due to the infection, undergo apoptotic cell death they could serve as a source of apoptotic bodies containing substantial amounts of the expressed antigen. Dendritic cells have been shown to take up apoptotic bodies and to efficiently present the enclosed antigens on MHC class I molecules in a process of so-called cross-priming[24]. In this respect rSFV, rVEE and rSIN differ in their natural cell tropism. While rVEE and rSIN readily transfect murine DCs[25-27], rSFV does not[20;28]. Studies with monocytes, macrophages and DCs from various origins, including human and murine DCs, revealed that rSFV has a very limited capacity to transfect these cell types *in vitro*[20]. To further investigate whether rSFV *in vivo* transfect professional antigen-presenting cells directly or whether the antigens reach these cells via cross-priming we compared the immunological effects of rSFV-constructs encoding different forms of Human Papillomavirus (HPV) E6 and E7 or influenza nucleoprotein[20;29]. These constructs differed in the amount of protein produced per cell or in the stability of the protein. We found that the induction of CTLs appeared to benefit from a large amount of stable antigen. In contrast, rapid antigen degradation, and thus availability of antigenic peptides in the transfected cell, was found to be disadvantageous. Based on these *in vitro* and *in vivo* results, we hypothesize that antigen presentation after SFV-based immunization proceeds via a mechanism in which antigen-presenting cells are not transfected directly but acquire antigen from other transfected cells and present it to CTLs in a process of cross-priming (Figure 6). Recently, Chen *et al.*,[30] confirmed

that infection of DCs with SFV *in vitro* is very inefficient. Interestingly, in this study on the role of MyD88 on the presentation of antigen derived from virally infected cells, these authors provide further proof that cross-priming indeed is the main mechanism by which immunity to an SFV replicon is generated.

Despite the difference in tropism for DCs between SIN and VEE, on the one hand and SFV on the other hand, immune responses elicited upon immunization with these recombinant alphaviruses in general is comparably efficient, yielding high levels of antigen-specific CTL and anti-tumour or anti-viral responses. As it is to be expected that rVEE and rSIN, similar to rSFV also infect cells other than DCs resulting in cross-priming of antigen, it remains to be established if the immune response elicited with rVEE and rSIN is a consequence of direct priming of DCs or of cross-priming or perhaps of a combination of both routes.

Wahlfors *et al.*,[31] evaluated the utility of both SIN and SFV vectors in comparison to each other and to other vector types on different target cells. In general, SFV appeared to have a higher transduction efficiency than SIN. However, high transduction efficiency turned out to be not necessarily accompanied by a high transgene expression: the rate of transgene expression was identical for both

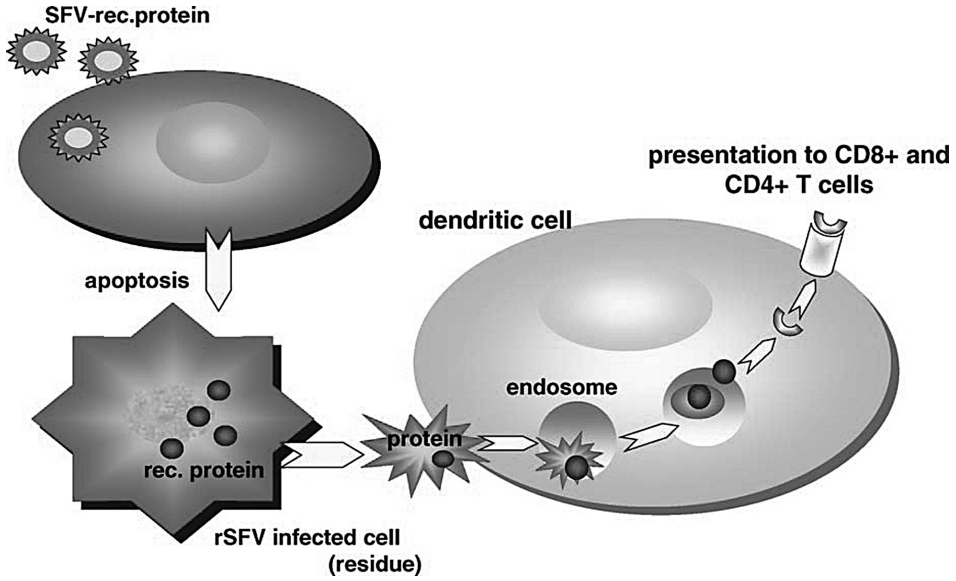

Figure 6. Schematic presentation of our hypothesis on the mechanism of antigen presentation after SFV-based immunization. Antigen-presenting cells (DC) are not transfected directly but acquire antigen from other transfected cells and present it to CD4+ and CD8+ T cells in a process of cross-priming. This process results in the generation of CTLs that can recognize tumour cells or virally infected cells expressing peptide epitopes of the recombinant protein as produced by rSFV.

viruses. These authors also compared alphaviral (SIN) vectors with adenoviral and retroviral vectors by transduction of several cell lines with these vectors carrying human clotting factor IX cDNA. The concentration of hFIX in the cell medium was followed for four days after transduction. SIN expression climbed rapidly to high level, followed by a quick drop, whereas retroviral and adenoviral expression initiated later and remained constant for a longer period of time (48h). This suggests that alphaviruses may be particularly valuable in situations that require rapid high-level but transient gene expression such as certain cancer gene therapies and vaccination.

Immunization strategies against infectious disease

The efficacy of rSFV expressing viral antigens has been evaluated in immunization studies in mice, guinea-pigs, monkeys and even in chicks. The antigens most extensively studied are the nucleoprotein and haemagglutinin of influenza virus[23], several HIV and SIV antigens[32;33], Human Papilloma virus (HPV) E6 and E7 protein[29;34-37], antigens from Louping Ill virus[38], Respiratory Syncytial virus[39;40], Tickborne Encephalitis virus[39], Hepatitis C virus[41] and Infectious Bursal Disease virus in chicks[42]. So far only a few studies have been reported on the use of rSFV immunizations against bacterial (*Chlamydia pneumoniae*[43] and *Brucella Abortus*[44]) and parasitic diseases (*Plasmodium Falciparum*[45;46]). Similarly, rSIN and rVEE have been studied as vectors for the induction of immune responses against HIV and SIV[47-50], HPV E7[51-54], Norwalk virus[55], Equine Arteritis virus in horses[56] *Anthrax*[57], *Staphylococcus*[58] and *Mycobacterium tuberculosis*[59].

These immunizations aim to induce sterilizing and long-lasting immunity against the microbe and/or eradication of infected cells by inducing micro organism-specific antibodies and/or specific CTL responses. Although in general T cell responses, including CTL responses, are readily induced against antigens encoded by recombinant alphaviruses, humoral responses against the antigens are not always induced. Humoral responses have been reported against rSFV-encoded nucleoprotein and hemagglutinin protein of influenza virus,[23] and spike proteins of Louping ill virus[38;60]. Yet, in our own studies on rSFV expressing HPV16 E6E7, in which we aim to induce strong CTL responses against HPV-infected cells, immunizations never resulted in detectable humoral responses against the E6 and E7 protein, using various E6E7 constructs and routes of immunization (as determined for us by Michael Pawlita, German Cancer Centre, Heidelberg, Germany). In a *Chlamydia pneumoniae* study[43] in which SFV-MOMP and SFV-Omp2 were evaluated, immunization resulted in detectable systemic Omp2 antibody levels while no MOMP-specific antibodies were induced. Despite this

difference in humoral response, both constructs induced similar antigen-specific T cell responses and similar levels of (partial) protection against a challenge with *Chlamydia pneumoniae*. Similarly, although T cell responses and (partial) protection were induced, no antibodies could be raised against the non-structural protein 3 of hepatitis C virus[41], SIV antigens[33] and *Brucella abortus* superoxide dismutase[44] encoded by rSFV and HIV-1 Gag encoded by rSIN[47].

Although for several immunization strategies the induction of humoral responses is not essential to confer protection as long as cellular immunity is induced, in the malaria studies of Chen *et al.*[46] the aim is to specifically induce antibodies that disrupt rosettes and protect against the sequestration of *Plasmodium falciparum*-infected erythrocytes. Therefore, these authors generated an SFV construct which generates proteins that are expressed extra-cellularly but anchored to the cell membrane by a transmembrane domain. In this way the antigen is displayed at the eukaryotic cell surface as is the native protein on the infected RBC surface. A prime-boost immunization regime of rSFV (prime) and recombinant protein (boost) resulted in antibody levels with rosette-disrupting activity.

In conclusion, the induction of humoral responses upon immunizations with alphaviral vectors varies a great deal depending on the antigen, the processing and presentation of the antigen and the immunization route. Nonetheless, in most studies, strong cellular immune responses are induced that result in (partial) protection against specific micro organisms. Further studies will have to elucidate if the humoral responses that in some models are induced against alphavirus-encoded antigens are indeed responsible for the observed protection or if cellular immunity that in general is also induced is the main effector mechanism.

Immunization strategies against tumors

Tumor vaccines based on alphaviruses are in general designed to stimulate or augment an immune response against existing tumor cells. The efficacy of rSFV has been evaluated in a limited number of preclinical tumor models, including melanoma (MAGE-3,[61] and mammary tumor (Neu)[62]). Yet, most of the work on the use of SFV vectors as tumor vaccines has concentrated on two mouse models of human tumors, which both are weakly antigenic and express known tumor-associated antigens. The first tumor model tested was the P815 mastocytoma tumor in mice[63,64], which expresses a weak tumor rejection antigen. Administration of rSFV expressing variants of the P815 antigen resulted in induction of strong cell-mediated immune responses against the tumor antigen. Vaccinated mice were protected against tumor challenge, or, when vaccinated therapeutically, showed inhibition of tumor growth or even total regression of the tumor. The second tumor

model used in alphaviral immunization studies is HPV-induced cervical cancer. In the next paragraph we will focus on our own studies using rSFV for therapeutic immunization against cervical cancer. Similar studies are being performed using rVEE, and rSIN as vectors[51;52].

Cervical cancer is the third most common cancer among women worldwide. It is caused by infection with high-risk Human Papillomavirus (HPV), in particular types 16, 18, 31, 33 or 45. High-risk HPVs have the capacity to transform cervical epithelial cells by integrating the open reading frames encoding the viral early proteins E6 and E7 into the host cell genome. This integration may lead to constitutive overexpression of E6 and E7, mediating transformation of the cells to a malignant phenotype. Since the continued production of E6 and E7 is required for the maintenance of the transformed phenotype, E6 and E7 in fact represent tumor-specific antigens in cervical carcinoma and premalignant HPV-transformed cells. As a consequence, E6 and E7 are potential targets for immunotherapeutic intervention strategies involving induction or stimulation of cytotoxic T lymphocyte (CTL) activity against HPV-transformed cells.

We initially demonstrated that immunization of mice with rSFV particles encoding HPV16 E6 and E7 resulted in a potent HPV16-specific CTL and anti-tumor response[34]. However, we were unable to induce full tumor protection. We next generated a construct in which the stop codon between E6 and E7 was removed and one base pair was inserted between the genes of E6 and E7 resulting in a construct that codes a stable fusion protein of E6 and E7. In addition, a translational enhancer was included to induce a high production of the fusion protein. The CTL response and anti-tumor activity induced by this so-called SFV-enhE6,7 virus appeared much stronger compared to the responses induced with rSFV, producing the separate E6 and E7 proteins[29]. Tumor treatment experiments, clearly demonstrated the high potency of the vector (Figure 7). Exponentially growing tumors of approximately 500 mm^3 in size were seen to completely resolve and even some tumors as large as 1500 mm^3 decreased to one third of their size[35;36]. Considering that a tumor nodule of 1000 mm^3 contains approximately 10^9 cells, this implies that in the latter situation, i.e. a tumor decreasing 1000 mm^3 in volume, the CTLs generated, manage to kill 10^9 cells in a one-week period. An other important aspect of our immunization approach is the induction of a long-term immune response, i.e. memory response is induced which even half a year after immunization mice can eradicate s.c. inoculated tumors.

Enhancement of CTL induction upon immunization with a vector encoding a more stable protein may seem inconsistent with several excellent studies in which MHC class I presentation has been demonstrated to be potentiated by enhanced

degradation of antigen. However, the explanation lies in the cross-priming pathway through which antigen is presented upon injection of rSFV, as described in a previous section. For cross-priming, the recombinant proteins to be presented should be stable for the time that is required for the entire process of infection of cells through uptake by APCs. Although RNA replication and translation occur within 6 hr after infection, dying of the infected cells by apoptosis takes another 24 to 72 hr. It therefore takes at least 24 hr after production before the protein gradually becomes available for APCs to be presented. Thus, the balance between stability and rate of degradation of the protein appears to determine the efficiency of antigen presentation.

In cervical cancer patients, HPV-specific CTL activity is generally low (Visser JTJ *et al.* 2005 Int J Cancer, in press), suggesting that they have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens. We recently studied whether SFV-expressing HPV16 E6 and E7 is potent enough to also prime a cellular immune response in immune-tolerant HPV-transgenic mice, in which CTL activity can not be induced using protein or DNA vaccines. We demonstrated that, depending on the route of immunization, SFV-enhE6,7 indeed has the capacity to induce HPV16 E7-specific cytotoxic T cells in HPV-transgenic mice[37]. Clearly, although the mechanism and kinetics of tolerance in this mouse model differ from that in humans, these studies demonstrate the potency of alphaviral vectors for immunization purposes.

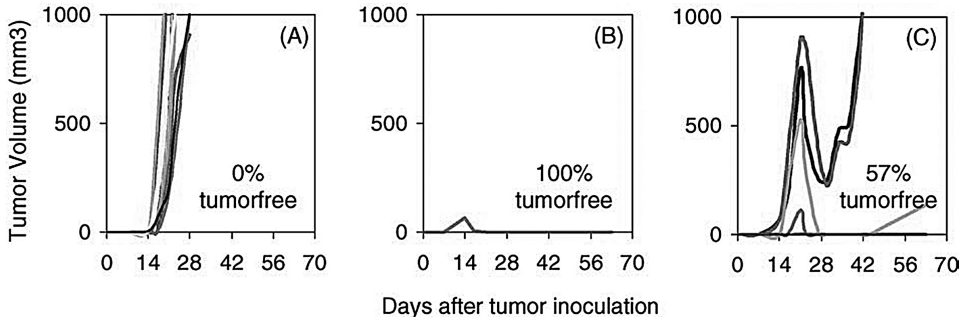

Figure 7. Efficacy of SFV-enhE6,7 immunization on regression of established tumours. Mice were inoculated s.c. in the neck with 2×104 TC-1 cells. Subsequently, mice were immunized and boosted i.v. with 5×106 SFV-enhE6,7 at days 7, 14 and 21 (n = 7; panel B), at days 14, 21, 28 (n = 7; panel C) after tumour inoculation.
As control, mice were injected i.v. with PBS (n = 10, panel A) at days 7, 14 and 21. Tumour measurements were initiated 10–14 days after tumour challenge and subsequently measured twice weekly. Given is the tumour volume of individual mice. The percentages indicate the percentage of tumour-free mice for each treatment at day 70 after tumour inoculation.

Clinical studies using alphavirus vectors

Until recently, alphavirus vector systems had not been used in human clinical studies. In 2003, a human trial among 40 volunteers has been performed in the area of HIV vaccine development (HIV Vaccine Trials Network (HVTN)). This study involved the use of a vector system based on VEE. The vaccine was well tolerated and no serious adverse events have been identified. In 2004/2005 another 96 volunteers were included in a multi centre dose-escalation study. Several rSFV applications will be evaluated in clinical trials in the near future. The European Vaccine Effort Against HIV/AIDS (Eurovac) will conduct human clinical studies using rSFV vectors encoding HIV-1 subtype C gag, pol, nef and env genes.

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CHAPTER 3

Eradication of established HPV16-transformed tumours after immunisation with recombinant Semliki Forest virus expressing a fusion protein of E6 and E7

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ABSTRACT

Previously, we described the efficacy of immunisation with recombinant Semliki Forest virus (SFV), expressing the human papillomavirus 16 (HPV) oncoproteins E6 and E7, in inducing HPV-specific CTLs and anti-tumour responses. Recently, we developed a novel recombinant SFV construct encoding a relatively stable fusion protein of HPV16 E6 and E7 under control of a translational enhancer derived from the SFV capsid protein. In the present study we demonstrate that immunisation of tumour-bearing mice with this improved vector results in the regression and complete elimination of established tumours. We furthermore demonstrate that a long-term high level of CTL activity, up to 340 days, accompanies the anti-tumour response. Thus, immunisation with recombinant SFV particles encoding increased levels of a fusion protein of HPV16 E6 and E7 efficiently induces CTL activity and CTL memory resulting in a potent therapeutic anti-tumour effect.

INTRODUCTION

After breast cancer, cervical cancer is the most common cancer in women worldwide. The association of high-risk human papilloma virus types (HPV) with cervical neoplastic lesions is very strong, independent of other risk factors. High-risk HPV prevalence in developed countries is as high as approximately 50% at an age of 20-30 years decreasing to less than 5% at an age of 50-60 years[1,2]. Yet, only a small fraction of HPV-infected women will eventually develop cervical cancer. Induction of cellular immunity seems to play an important role in clearing the HPV infection, as immunocompromised individuals are at increased risk of anogenital neoplasia[3]. Although cellular and humoral responses against HPV antigens can be observed in patients with pre-invasive and invasive cervical carcinoma lesions, these responses apparently do not suffice to effectively eliminate HPV and HPV-transformed cells[4]. Several studies have shown that immunisation with the early protein E7 of HPV 16 results in the induction of CTL and anti-tumour responses in murine tumour models. In general, vector-based vaccines result in stronger immune responses compared to immunisations using whole protein or peptide emulsified in adjuvants[5,6,7]. We are exploiting an alphavirus vector system based on Semliki Forest virus (SFV) to induce a cellular immune response against HPV-transformed tumour cells[8]. In a previous study in mice, we have demonstrated that indeed an HPV-specific immune response can be induced upon administration of recombinant SFV expressing HPV16 E6 and E7[9]. Subsequently, we generated a novel construct encoding both a translational enhancer and a fusion protein of E6 and E7[10]. Infection of cells with this SFV vector (SFV-enhE6,7) gives rise to the enhanced production of a fusion protein of E6 and E7. The fusion protein is more stable than the E6 and E7 proteins separately as demonstrated by pulse-labelling experiments. Immunisation of mice

with SFV-enhE6,7 resulted in more potent CTL responses compared to immunisation with SFV expressing the separate proteins. In the present study we determined whether the enhanced immune response observed upon SFV-enhE6,7 immunisation is able to eradicate pre-existing tumours. Furthermore, we determined the effect of immunisation with SFV-enhE6,7 on long-term tumour protection and long-term CTL activity.

MATERIALS AND METHODS

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (# CCL-10). The cells were grown in GMEM (Life Technologies, Paisley, UK) containing 5% foetal calf serum (PAA laboratories, Linz, Austria). C3 cells, 13-2 cells and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). The C3 cell line was derived from C57BL/6 (H-2^b) embryonic cells transfected with a plasmid containing the complete HPV16 genome[11]. The 13-2 cell line was generated from C57Bl/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF)[12]. The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras[13]. C3, 13-2 and TC-1 cells were grown in IMDM (Life Technologies) supplemented with 10% foetal calf serum. Both media contained penicillin and streptomycin (Life Technologies; 100 U/ml and 100 μg/ml, respectively).

Mice

Specific-pathogen-free female C57Bl/6 mice (Harlan CPB, Zeist, The Netherlands) were between 6 and 10 weeks of age at the start of the immunisation protocols.

Production and purification of recombinant SFV particles

pSFV-Helper 2 was kindly provided by Dr. P. Liljeström (Karolinska Institute, Stockholm, Sweden). pSFV3 was obtained from Life Technology. The HPV16 E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7, which was kindly provided by Dr. J. Ter Schegget (Amsterdam Medical Center, Amsterdam, The Netherlands)[14]. In this plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. The construction of PSFV3-enhE6,7 is described in detail before[10]. In short, in pSFV3-enhE6,7 one base pair is inserted between E6 and E7 and the stop codon TAA of E6 is changed in GAA while furthermore a sequence encoding a

translational enhancer is cloned in front of the E6,7 fusion construct. Thus, pSFV3-enhE6,7 encodes an enhanced expression of a fusion product of E6 and E7.

The pSFV3-enhE6,7, pSFV3-LacZ (Life Technologies) and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit and linearised by digestion with SpeI (Life Technologies). RNA was synthesised from the linearised DNA by *in vitro* transcription using SP6 RNA polymerase (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Capping analogue was obtained from Life Technologies. Fifteen μg SFV3-enhE6,7 or SFV3-LacZ and 7.5 μg SFV-Helper 2 RNA were admixed and cotransfected into $8x10^6$ BHK cells in 0.8 ml GMEM by electroporation using the Biorad Gene Pulser^{R}II (two pulses of 850 V/ 25 μF; Biorad, Hercules, CA, USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured for 36 hr at 37°C and 5% CO_2. The medium, containing the SFV-E6E7 or SFV-LacZ particles was centrifuged twice in a JA 20 rotor (Beckman, St. Paul, MN, USA) at 1800 rpm (i.e. 40,000xg at r_{max}) to remove cells and cellular debris.

The SFV particles were purified on a discontinuous sucrose density gradient (2 ml of a 15% sucrose solution (w/v) and 1 ml of a 50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1mM EDTA, pH 7.4)). Virus was collected from the interface. Sucrose was removed from the virus solution by overnight dialysis against TNE-buffer. The virus suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in N_2 and stored in aliquots at -80°C.

Before use, SFV particles were incubated with 1/20 volume of α-chymotrypsin (10 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature to cleave the mutated viral E2 spike protein. Subsequently, α-chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ ml; Sigma Chemical Co.).

Titer determination of SFV particles

Recombinant SFV particles were titrated by serial dilution on monolayers of BHK cells. After infection and overnight incubation the cells were fixed for 10 minutes in 10% acetone and stained using a polyclonal rabbit anti-replicase (nsP3) antibody (a kind gift from Dr T. Ahola, Biocentre Viiki, Helsinki, Finland) as primary antibody and FITC-labelled goat-anti-rabbit IgG as a secondary antibody (Southern Biotech. Ass., Birmingham, AL, USA). Positive cells were counted and the titer was determined after correcting for the dilution factor and the dilution caused by the activation and the volume of particles added.

Tumour treatment experiments

Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Life Technologies). Subsequently, mice were immunised and boosted s.c. in the flank with $5x10^6$ SFV-enhE6,7 particles, $5x10^6$ SFV-LacZ particles or phosphate-buffered saline (PBS, pH 7.4) starting at days 2, 7 or 14 after tumour inoculation. Tumour measurements were always perfpormed blindly by the same skilled technician. At a tumour volume of approximately 1000 mm^3, the mice were sacrificed.

Mice that cleared the tumour in the tumour treatment experiments as described above were rechallenged s.c. in the neck with $2x10^4$ TC-1 cells three months after the initial tumour challenge without additional immunisations. Since all control PBS-treated mice developed a tumour upon the initial tumour challenge, in the rechallenge experiments age-controlled naive mice were included.

CTL assay

At several time points after s.c. immunisation in the flank and/or s.c. tumour inoculation in the neck, spleen cells were isolated and cocultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm^2 culture flasks, placed upright. After one week in culture, cells were harvested and a CTL assay was performed by a standard 4-hr ^{51}Cr release assay in triplicate determinations. Target cells were labeled for 1 h with 3.7 MBq $^{51}Cr/10^6$ cells in 100 μl medium (^{51}Cr was from Amersham, London, UK). The mean percentage of specific ^{51}Cr-release of triplicate wells was calculated according to the formula: % specific release = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] cpm x 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

RESULTS

Therapeutic immunisation against HPV transformed tumours with SFV-enhE6,7

In a previous study we demonstrated that immunisation of mice with SFV-enhE6,7 resulted in strong, long-lasting HPV-specific CTL responses as determined in bulk CTL assays and CTL precursor frequency assays (Interferon-gamma Elispot)[10]. We furthermore demonstrated that immunisation with SFV-enhE6,7 particles results in prevention of tumour outgrowth and subsequent protection against tumour re-challenge. In the present study we determined the efficacy of SFV-enhE6,7 in the eradication of established tumours. Mice were inoculated s.c. in the neck with $2x10^4$

TC-1 cells and subsequently immunised with SFV-enhE6,7 particles. All control mice, either injected s.c. with PBS or with recombinant SFV expressing LacZ, developed tumours within 14 days after tumour inoculation (Figure 1, panels A and B; Figure 2). In 40% of mice immunised and boosted on days 2 and 7 with SFV-enhE6,7 a small tumour nodule, less than a pin-head, could be felt (Figure 1, panel C; Figure 2). These mice were boosted on day 14 and from day 17 on all mice became and remained tumour-free until three months after tumour inoculation at which time point the mice were rechallenged, as described below. All mice immunised s.c. on days 7, 14 and 21, developed a palpable tumour at day 14 after tumour inoculation. At day 21, in 87% of the mice no tumour could be palpated anymore. Ultimately, 13 of 21 mice were tumour-free three months after tumour inoculation (Figure 1, panel D). When immunisation was initiated on day 14 after tumour inoculation tumours initially grew very fast, comparable to tumours in control mice. However, upon immunisation, tumours as large as 650 mm^3 regressed to undetectable levels (Figure 1, panel E). At later time point, as late as 10 weeks after inoculation, some of these undetectable tumours started to grow again. Ultimately, in this group of mice 2 of 6 mice were tumour-free.

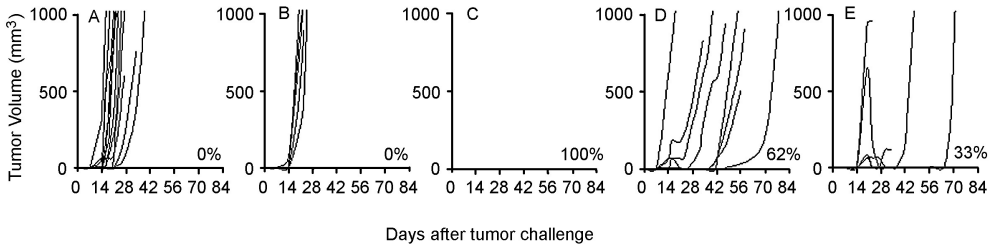

Figure 1. Growth and regression of HPV-transformed tumours upon treatment with SFV-enhE6,7. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, mice were immunised and boosted s.c. with $5x10^6$ SFV-enhE6,7 particles at days 2, 7 and 14 (n=7; panel C) at days 7, 14 and 21 (n=21; panel D) or at days 14, 21, 28 (n=6; panel E) after tumour inoculation. As control, mice were injected s.c. with $5x10^6$ SFV-LacZ particles (n=5; panel B) or with PBS (n=13, panel A) at days 2, 7 and 14. Tumour measurements were initiated 10-14 days after tumour challenge and subsequently measured twice weekly. Shown are the results of three separate experiments. Given is the tumour volume of each individual mouse. The percentages indicate the percentage of tumour-free mice for each treatment at day 84 after tumour inoculation. At a tumour volume of approximately 1000 mm^3, the mice were sacrificed.

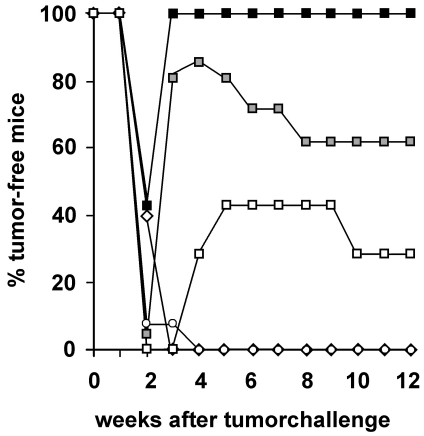

Figure 2. Treatment of established HPV-transformed tumours with SFV-enhE6,7. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, mice were immunised and boosted s.c. with $5x10^6$ SFV-enhE6,7 particles at days 2, 7 and 14 (n=7; black squares), 7, 14 and 21 (n=21; grey squares) or 14, 21, 28 (n=6; white squares) after tumour inoculation. As a control, mice were injected s.c. with $5x10^6$ SFV-LacZ particles (n=5; open diamonds) or PBS (n=13; open circle) at days 2, 7 and 14. Tumour measurements were initiated 10-14 days after tumour challenge and subsequently measured twice weekly. Shown are the results of three separate experiments. Given is the percentage of tumour-free mice.

Mice are protected against a tumour rechallenge 3 months after the initial challenge and immunisation with SFV-enhE6,7.

In one experiment, all mice that were tumour-free after the initial challenge, were rechallenged three months later without additional immunisations. Since all control mice initially challenged developed a tumour, age-matched control mice were included in the rechallenge experiment. All control mice again developed a detectable tumour within 14 days after tumour inoculation (Figure 3). Twelve of thirteen mice that were entered in the rechallenge experiment did not develop a detectable tumour up to 12 weeks after rechallenge, i.e. 25 weeks after the first tumour challenge. In contrast to the first tumour challenge, when most mice, dependent on the time of initiation of immunisation, developed a detectable tumour within 14 days after tumour cell inoculation, no tumours were detected at this time point in the rechallenged mice.

The memory CTL response as observed above might be blurred by the fact that these mice were not only immunised with SFV-enhE6,7 but had also been challenged once or twice with TC-1 tumour cells. We therefore performed a control experiment in which CTL activity was determined in mice that had been immunised with SFV-enhE6,7 particles and/or challenged with $2x10^4$ TC-1 tumour cells. Two groups of each three mice were inoculated with tumour cells on day 0. Fourteen days after

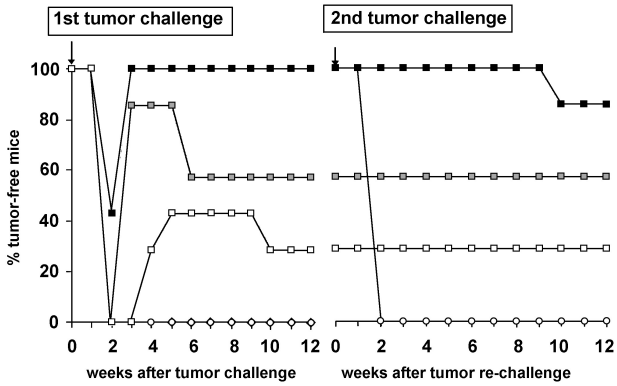

Figure 3. Effect of immunisation with SFV-enhE6,7 on a tumour-rechallenge 12 weeks after the initial tumour challenge and immunisation. Mice that cleared the tumour in one of the tumour treatment experiments as described in figure 2 were rechallenged with $2x10^4$ TC-1 cells three months after the initial tumour challenge without additional immunisations. Since all control PBS-treated mice developed a tumour upon the initial tumour challenge, in the rechallenge experiments age-controlled naive mice (n=4) were included. Given is the percentage of tumour-free mice.

inoculation all mice had developed a small tumour. One group was then immunised once with $5x10^6$ SFV-enhE6,7 particles s.c. the other group was injected s.c. with PBS. The third group of three mice was injected on day 0 with PBS and on day 14 with $5x10^6$ SFV-enhE6,7 particles s.c.. As a control one mouse was injected with PBS on days 0 and 14. On day 21 tumour volumes were determined in mice inoculated with tumour cells and spleens from all mice were isolated to perform a CTL assay. The CTL responses in non-tumour bearing mice ranged between 40% and 65% at an effector to target ratio of 30:1. In mice inoculated with tumour cells without immunisation no CTL activity was detectable, strongly suggesting that the tumours which were between 65 and 1414 mm^3 at the time of CTL analysis do not induce an HPV specific CTL response. In addition, tumour growth does not potentiate the CTL response already induced upon immunisation with SFV-enhE6,7 as the percentages of tumour cells lysis of tumour-bearing immunised mice were as high as those obtained in mice immunised only.

Memory CTL activity in mice immunised with SFV-enhE6,7 particles and (re)challenged with tumour cells.

The observation that none of the immunised mice rechallenged 3 months after immunisation developed a small, but detectable tumour nodule two weeks after rechallenged suggests that HPV-specific memory CTLs or CTL precursors are still present at this time point. A fast and powerful antitumour response is apparently able to kill rechallenged tumour cells in a short time period. In a next experiment

Table 1. CTL activity as determined up to 340 days after immunisation with SFV-enhE6,7

Dose[a]	*Day of tumour inoculation*	*Days of immunisation*	*Day of tumour challenge*	*Day of tumour re-challenge*	*Day of CTL analysis*[b]	*% specific cytolysis (number of mice)*[c]
$1x10^6$		0-10-22	29	200	340	61 (1)
$1x10^6$		0-15-31	38	130	270	64 (2)
$5x10^6$		0-15-31	38	130	270	61 ± 2 (3)
$5x10^6$	0	2-7-14		93	184	52 ± 12 (5)
$5x10^6$	0	7-14-21		93	184	63 ± 2 (4)
$5x10^6$	0	14-21-28		93	184	50 (2)
$5x10^6$	0	7-14-21		-	140	64 ± 1 (3)

[a]Mice were immunised s.c. and boosted twice s.c. with purified $1x10^6$ or $5x\ 10^6$ SFV-enhE6,7 either before tumour (re)challenge or after tumour inoculation according to the scheme given above.
[b]CTL activity was determined 140 to 340 after the initiation of the experiment. After 7 days in vitro restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay.
[c]Shown are the mean levels of cytolysis and standard deviation of the indicated number of mice tested, at an effector to target ratio of 30 to 1. Since none of the control mice remained tumour free, two control naive mice were included in the CTL experiment. In these mice no CTL activity was detectable (not shown)

we therefore studied the CTL response in mice that were tumour free either in a preimmunisation experiment (in which mice were immunised before tumour inoculation)[10] or in tumour treatment experiments. Since initially these were separate experiments the time-point of the CTL determination ranges between 140 and 340 days after immunisation. In Table 1, the immunisation/tumour inoculation schemes are shown of the mice assayed for CTL activity. Independent on the immunisation scheme or dosage used, up to 340 days after immunisation a high level of CTL activity was still detectable in spleen cells.

DISCUSSION

Here were demonstrate the efficacy of recombinant SFV-enhE6,7 in inducing a potent anti-tumour response. The experiments were conducted using TC-1 cells as developed by Wu and colleagues[13]. The model is very reproducible resulting in the development of s.c. growing tumours in all control mice within 2 weeks after tumour inoculation. In forty percent of mice immunised with SFV-enhE6,7 two days after tumour inoculation and boosted on days 7 and 14, small tumour nodules could initially (days 14-21) be palpated. Yet, the nodules disappeared in time and all mice remained tumour-free thereafter. The other sixty percent of mice eliminated the tumour already prior to the initiation of tumour palpation (day 14) and also remained tumour-free. Thus, immunisation resulted in rapid CTL induction able to efficiently eliminate the fast-growing tumour. Tumour rechallenge of the mice demonstrated that 3 months after immunisation the immune response sufficed to protect the mice from tumour growth. The decreased efficiency of the late immunisation regimen (immunisation initiated 7-14 days after tumour inoculation) compared to the early immunisation regimen is can presumably be ascribed to the very fast growth of the tumour. Although most of the tumours initially regress, tumour growth at this time-point outpaces the immune response, which has not reached its full strength at this point. Notably, all mice that eradicated the tumour when immunisation was initiated 7 days after tumour inoculation, remained tumour-free upon a second tumour challenge 3 months later. In addition, CTL responses determined at very late time points after immunisation and tumour challenge demonstrate that up to 11 months after immunisation high levels of CTL activity could be determined.

In recent years several murine studies have been described demonstrating the *in vivo* anti-tumour efficacy of several immunisation strategies against HPV-transformed tumours. While in most of these studies mice were immunised prior to tumour inoculation[12,14-18], only few studies report on therapeutic anti-tumour responses[19-23]. Partial prophylactic protection against tumour outgrowth was obtained upon immunisation with protein/adjuvant preparations, vaccinia virus and adenovirus-based vaccines. When this manuscript was in preparation, results were published on the efficacy of another alphavirus vector system, Venezuelan Equine Encephalitis (VEE)[24]. Although the HPV tumour model used (C3 cells) differed from our tumour model system, the results were comparable to our results with SFV-enhE6,7.

Alphavirus expression systems based on suicidal virus particles derived from SFV, VEE and Sindbis virus efficiently deliver heterologous genes, eliciting broad tumour-specific immune responses in animals, including primates[24-29]. Alphavirus

vectors are interesting and promising vectors since they infect a broad host range of cell types and there is no pre-existing immunity against the vector in the majority of individuals. The strong immune responses induced against heterologous proteins expressed by these suicidal vectors is most likely elicited by cell death induced upon infection followed by the release of apoptotic bodies that can be taken up by antigen-presenting cells, thus enhancing cross-priming[30,31].

In the present study we used an SFV vector expressing (SFV-enhE6,7) a fusion construct of E6 and E7 under control of a translational enhancer derived from the SFV capsid protein. As described (10), this construct elicits a much stronger immune response than a construct encoding E6 and E7 as separate proteins in the absence of the translational enhancer. We hypothesised that, due to the specific properties of the fusion protein and the elevated expression levels, SFV-enhE6,7 gives rise to a significantly improved processing and presentation of the antigen to immune effector cells. As mentioned above, upon infection with the SFV vector the recombinant antigen is most likely presented to the immune system in a process of cross-priming. Cellular debris from infected cells taken up by an APC will result in processing and presentation by both MHC class I and class II molecules. Thus, the recombinant proteins to be presented should be stable for the time that is required for the entire process of infection of cells through MHC presentation by APCs. Indeed we have demonstrated that the E6,7 fusion protein is more stable than the individual E6 and E7 proteins. Furthermore in the presence of the translational enhancer, which results in an approximately 10-fold increased production of recombinant protein per infected cell is expected to strongly facilitate CTL induction through cross-priming. Yet, since 100- to 1000-fold less SFV-enhE6,7 particles suffice to elicit responses comparable to those observed with SFV-E6E7, the enhanced immune response of SFV-enhE6,7 is presumably not merely due to the an increased level of protein production per cell[10].

Modification of the E7 protein has been shown in several studies to significantly enhance the immune responses elicited. Although based on another processing mechanism, Lin *et al.*[13] demonstrated that therapeutic responses were not obtained using vaccinia virus encoding unmodified E7, while a recombinant vaccinia vector in which E7 was linked to the sorting signals of lysosome-associated membrane protein was able to elicit anti-tumour responses. In addition, a fusion protein of BCG-hsp65 and HPV16 E7 was able to induce regression of TC-1 tumours while an admixture of hsp65 and E7 did not induce significant tumour regression[20]. It should be noted, however, that in the recently published VEE study unmodified E7 was expressed[24]. Although we have demonstrated that also SFV expressing unmodified E6 and E7 elicited prophylactic immune responses[9], the responses induced upon immunisation

with VEE encoding unmodified E7 seem stronger, although not stronger than the results observed with SFV-enhE6,7. This apparent difference between comparable vector systems might be due to the fact that the envelope glycoproteins of VEE, in contrast to those of SFV, confer dendritic cell tropism to the vector[32]. This would imply that processing and presentation of heterologous proteins expressed by VEE does not require cross-priming, thus possibly explaining the apparent superior efficiency of this vector with regard to presentation of E7. A potential problem associated with direct antigen expression in dendritic cells lies in the fact that infection with alphaviruses results in cell death by apoptosis giving the dendritic cells only a short time-period to present the heterologous protein. An additional drawback might be that, while cross-priming results in both MHC class I and class II presentation of the antigen, thereby eliciting cytotoxic ánd helper T cell responses, transfection of dendritic cells in general only results in MHC class I presentation of antigen. It should be noted that CD4+ T helper responses are important for the maintenance of CD8+ T cell numbers and long-term memory responses33-35. Moreover, Marzo *et al.* demonstrated that CD4+ T cells are required for CD8+ T cell infiltration of a tumour[35].

Optimal immune responses are believed to be essential to overcome immune evasion by the tumour and/or to break immune tolerance against tumour antigens. Here we demonstrate that immunisation with SFV expressing a fusion protein of HPV E6 and E7 results in a potent and long-term memory CTL response that correlates with a potent anti-tumour effect. From a clinical point of view as well as from a scientific point of view, comparative studies on the efficacy of different vectors systems should be performed. The level of the immune response, the duration of the immune response and very importantly the safety of the vector system will be critical to ultimately develop the most optimal immunisation strategy against cervical cancer.

Acknowledgements

We thank Prof. Dr. A.G.J. van der Zee and Dr. Anke Huckriede for advise and stimulating discussions and Marijke Holtrop for generating the SFV-enhE6,7 construct.

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CHAPTER 4

Superior therapeutic efficacy of alphavirus-mediated immunization against human papilloma virus type 16 antigens in a murine tumor model: effects of the route of immunization

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Antivir Ther. 2004 Oct;9(5):733-42

ABSTRACT

In our efforts to develop a strong, effective immune response against cervical carcinoma and premalignant disease we study the use of recombinant Semliki Forest virus (SFV) encoding the oncoproteins E6 and E7 from high-risk human papilloma viruses (HPV). Optimal immunization conditions are required for immunotherapeutic treatment of cervical cancer as it has been postulated that cervical cancer patients are immune-suppressed and/or immunologically tolerant for HPV. We previously generated an optimized construct encoding a fusion protein of HPV16 E6 and E7 and a translational enhancer (enhE6,7). Immunization of mice with SFV-enhE6,7 was shown to induce CTL responses and resulted in the eradication of established tumors. We now demonstrate, using HPV16-specific MHC class I tetramers, that high pCTL frequencies can be induced. Yet, this induction is strongly influenced by the route of immunization applied. While in bulk CTL assays, requiring *in vitro* restimulation, CTL activity can be observed upon s.c., i.p., i.v. and i.m. immunization, detectable pCTL frequencies, without *in vitro* restimulation, are only induced upon i.m. and i.v. immunization. The route of immunization also strongly influences the dose of viral vector needed to induce CTLs and tumor therapy. As few as $5x10^4$ SFV-enhE6,7, primed and boosted i.v., are needed to eradicate tumors in 6 of 7 mice treated. Furthermore, exponentially growing tumors of approximately 500 mm^3 in size were seen to completely resolve and even tumors as large as 1500 mm^3 decreased to one third of their size. Apart from this potency, SFV vectors can safely be used for the expression of oncoproteins such as E6 and E7, since the viral RNA is not integrated in the host genome. Thus, SFV-enhE6,7 meets with the criteria that a vaccine against cervical cancer should be safe and induce a very strong, long-lasting CTL response, strong enough to eradicate existing tumors.

INTRODUCTION

Cervical cancer is a virus-induced cancer. Infection of the cervical epithelia with so-called high-risk human papillomavirus (HPV) may lead to cell transformation and oncogenesis[1]. Upon integration of part of the HPV genome in the host cell, virally encoded oncogenic proteins are produced that can bind to the tumor suppressor proteins p53 and pRb resulting in deregulation of the cell cycle control and ultimately cell transformation[2].

At present, several promising clinical studies are being performed studying the effectiveness of prophylactic vaccines based on HPV virus-like-particles (VLPs) composed of one or both viral structural proteins, L1 and L2[3]. The aim of these prophylactic strategies is to induce neutralizing antibodies that can prevent infection of cells with HPV. One of the problems associated with the development of a prophylactic vaccine against HPV is that it should be composed of at least 15 different HPV VLP types to prevent approximately 95% of the HPV-induced cervical lesion[4]. Despite the promising results, and even if all problems associated with the development of a fully protective prophylactic vaccine are solved and

worldwide children are vaccinated, women that have already been infected before vaccination are at risk to develop (pre)malignant lesions or cervical cancer. Since the development of HPV-induced lesions in general manifests 10-20 years after infection this will concern many women.

In contrast to prophylactic vaccines, a therapeutic vaccine against cervical cancer is aimed at the induction of a cellular immune response directed against tumor cells. The malignant phenotype of a HPV-transformed tumor cell requires the continued expression of the oncoproteins E6 and E7, making them attractive targets for T cell mediated immune responses[5]. Therapeutic immunization approaches that are nowadays being investigated can roughly be divided in protein/peptide-based strategies and vector-based strategies[6]. We are developing a therapeutic genetic vaccine against HPV-transformed cells based on Semliki Forest virus (SFV), a virus belonging to the alphavirus genus. Vectors based on alphaviruses are gaining increasing interest for their superiority over other viral vectors with respect to the induction of cellular immune responses. Recombinant SFV particles (rSFV) carry the RNA that codes for the replicase and the heterologous gene but lack RNA that codes for the structural proteins[7;8]. Consequently, infection of cells with rSFV will not result in productive replication and the virus will not spread beyond initially infected cells. We recently demonstrated, that cross-priming evokes the immune response induced upon SFV immunization[9]. Infection of cells with rSFV results in a high production of recombinant protein by infected cells that, due to the SFV infection, will die through apoptosis. Professional antigen-presenting cells (APC) that take up these apoptotic cells or apoptotic residues will process the recombinant protein for MHC class I and II presentation, resulting in the activation of specific cytotoxic T cells (CTL) and helper T cells.

We previously demonstrated that s.c. immunization of mice with rSFV encoding a fusion protein of E6 and E7 and a translational enhancer[10] (SFV-enhE6,7) resulted in strong, long-lasting HPV-specific CTL responses, as determined in bulk CTL assays and Interferon-gamma Elispot assay, and in *in vivo* anti-tumor responses[11;12]. Colmenero *et al.*[13] reported on the localization of viral RNA after injecting rSFV via different injection routes. It was demonstrated that upon i.v. injection, rSFV-RNA was distributed to a variety of different tissues whereas it was confined more locally after i.m. and s.c. injections. Upon i.v. and i.m. injection, but not upon s.c. injection, rSFV-RNA could be detected in spleen. Morris-Downes *et al.*[14] studied the distribution and persistence of i.m. injected rSFV in mice and chickens and demonstrated that i.m. injected rSFV showed persistence at the injection site up to 7 days, transient detection in secondary lymphoid organs and no dissemination to distal sites. We reasoned that these differences in gene

expression may influence the type and magnitude of the immune response. Therefore, in the present study we determined the efficacy of the i.m. and i.v. route of immunization. While, as reported previously, SFV-enhE6,7 injected s.c. already evokes a potent immune and anti-tumor response, we now demonstrate that these responses are strongly enhanced upon i.m. or i.v. injection. Rapidly growing tumors up to 1000 mm^3 are minimized or even completely eradicated.

MATERIALS AND METHODS

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (# CCL-10). The cells were grown in GMEM (Invitrogen, Breda, The Netherlands) containing 5% fetal calf serum. 13-2 cells and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). The 13-2 cell line was generated from C57Bl/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF)[15]. The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras[16]. 13-2 and TC-1 cells were grown in IMDM with Glutamax-I (Invitrogen) supplemented with 10% fetal calf serum and penicillin and streptomycin (Invitrogen; 100 U/ml and 100 μg/ml, respectively). TC-1 medium was furthermore supplemented with non-essential amino acids (Invitrogen; 100-fold dilution), sodium pyruvate (Life Technologie; 1 mM) and Geneticin G418 Sulphate (Roche, Germany; 5mg/ml).

Mice

Specific-pathogen-free female C57Bl/6 mice (Harlan CPB, Zeist, The Netherlands) were between 6 and 10 weeks of age at the start of the immunization protocols.

Production and purification of rSFV

Dr. P. Liljeström (Karolinska Institute, Stockholm, Sweden) kindly provided pSFV-Helper 2. pSFV3 was obtained from Life Technology. The HPV16 E6 and E7 genes were obtained from the plasmid pRSV-HPV16E6E7, which was kindly provided by Dr. J. Ter Schegget (Amsterdam Medical Center, Amsterdam, The Netherlands)[17]. In this plasmid the HPV16 E6 and E7 genes are present in tandem, with a stop codon after the E6 gene. The construction of PSFV3-enhE6,7 is described in detail before[11]. In short, in pSFV3-enhE6,7 one base pair is inserted between E6 and E7 and the stop codon TAA of E6 is changed in GAA while furthermore a sequence encoding a

translational enhancer is cloned in front of the E6,7 fusion construct. Thus, pSFV3-enhE6,7 encodes an enhanced expression of a fusion product of E6 and E7. The pSFV3-enhE6,7 and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit and linearized by digestion with SpeI (Invitrogen). RNA was synthesized from the linearized DNA by *in vitro* transcription using SP6 RNA polymerase (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Capping analogue was obtained from Invitrogen. Fifteen μg SFV3-enhE6,7 and 7.5 μg SFV-Helper 2 RNA were admixed and cotransfected into $8x10^6$ BHK cells in 0.8 ml GMEM by electroporation using the Biorad Gene Pulser^{R}II (two pulses of 850 V/ 25 μF; Biorad, Hercules, CA, USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured for 36 hr at 37°C and 5% CO_2. The medium, containing the SFV-enhE6,7 particles was centrifuged twice in a JA 20 rotor (Beckman, St. Paul, MN, USA) at 1800 rpm (i.e. 40,000xg at r_{max}) to remove cells and cellular debris.

The SFV particles were purified on a discontinuous sucrose density gradient (2 ml of a 15% sucrose solution (w/v) and 1 ml of a 50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1mM EDTA, pH 7.4)). Virus was collected from the interface. Sucrose was removed from the virus solution by overnight dialysis against TNE-buffer. The virus suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in N_2 and stored in aliquots at -80°C.

Before use, SFV particles were incubated with 1/20 volume of α-chymotrypsin (10 mg/ml; Sigma Chemical Co., St. Louis, MO, USA) for 30 min at room temperature to cleave the mutated viral E2 spike protein. Subsequently, α-chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ ml; Sigma Chemical Co.).

Titer determination of rSFV

SFV particles were titrated by serial dilution on monolayers of BHK cells. After infection and overnight incubation the cells were fixed for 10 minutes in 10% acetone and stained using a polyclonal rabbit anti-replicase (nsP3) antibody (a kind gift from Dr T. Ahola, Biocentre Viiki, Helsinki, Finland) as primary antibody and FITC-labelled goat-anti-rabbit IgG as a secondary antibody (Southern Biotech. Ass., Birmingham, AL, USA). Positive cells were counted and the titer was determined after correcting for the dilution factor and the dilution caused by the activation and the volume of particles added.

Immunizations

For in CTL analysis, mice were immunized s.c., i.v., i.m or i.p. with 10^3 to $5x10^6$

SFV-enhE6,7, followed by one booster immunization with a two-week interval. As negative controls, mice were injected with PBS.

CTL assay

Seven to ten days after immunization, spleen cells were isolated and cocultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm^2 culture flasks, placed upright. After one week in culture, cells were harvested and a CTL assay was performed by a standard 4-hr ^{51}Cr release assay in triplicate determinations. Target cells (13-2 cells) were labeled for 1 h with 3.7 MBq $^{51}Cr/10^6$ cells in 100 μl medium (^{51}Cr was from Amersham, London, UK). The mean percentage of specific ^{51}Cr-release of triplicate wells was calculated according to the formula: % specific release = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] cpm x 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

MHC class I tetramer staining and FACS analysis

To analyze the number of $CD8^+$ T cells specific for the HPV 16 $E7_{49-57}$ peptide RAHYNIVTF we used K^b-RAHYNIVTF tetramers produced in the laboratory of Dr. Ton Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Spleen cells were washed with FACS buffer (PBS containing 0,5% BSA and 0,02% sodium azide) and stained with FITC-conjugated anti-CD8a (Pharmingen) together with PE-conjugated K^b-RAHYNIVTF tetramers for 20 minutes at 4°C. Spleen cells were washed three times and analyzed by flow cytometry (ELITE, Coulter). Live cells were selected based on propidium iodide exclusion.

Tumor treatment experiments

Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Invitrogen). Subsequently, mice were immunized and boosted twice with a one-week interval, with SFV-enhE6,7 or phosphate-buffered saline (PBS, pH 7.4) starting at days 7, 14 or 17 after tumor inoculation. Immunization routes were s.c., i.p., i.v. or i.m. The same skilled technician always did tumor measurements. At a tumor volume of approximately 1000 mm^3, the mice were sacrificed. Mice that cleared the tumor in the tumor treatment experiments as described above were rechallenged s.c. in the neck with $2x10^4$ TC-1 cells six months after the initial tumor challenge without additional immunizations. Since all control PBS-treated mice developed a tumor upon the initial tumor challenge, in the rechallenge experiments naive mice were included.

RESULTS

Influence of the route of immunization on the induction of CTL responses

In a previous study we demonstrated that s.c. immunization of mice with SFV-enhE6,7 resulted in HPV-specific CTL responses as determined in bulk CTL assay and Interferon-gamma Elispot assay[11]. We now compared CTL activity and precursor CTL (pCTL) frequencies induced upon s.c. immunization with the responses induced after i.v, i.m. or i.p. immunization. Mice were immunized and boosted once with 10^6 SFV-enhE6,7, CTL activity was determined one week after the booster using a standard bulk CTL-assay of spleen cells stimulated for 6 days with irradiated TC-1 cells. As shown in Figure 1, apart from one mouse in the group immunized s.c., the bulk CTL analysis did not demonstrate significant differences between the four routes of immunization tested. Yet, the frequency of pCTLs, determined with MHC class I tetramers refolded with HPV16 $E7_{49-57}$ peptide, revealed that mice immunized i.v. and i.m. had higher frequencies of pCTLs compared to mice immunized i.p. or s.c. (Figure 2). Closer examination of the bulk CTL results (Figure 1) reveals that indeed the CTL levels of mice immunized i.v. and i.m. at an effector to target ratio of 3:1 are higher than those of mice immunized s.c. or i.p..

We next questioned whether these differences are also reflected in the minimal effective dose of SFV. Mice were immunized with decreasing doses of SFV-enhE6,7 either i.v. or s.c. (Figure 3). 10^6 (Figure 3A, squares) and 10^5 SFV-enhE6,7 (Figure

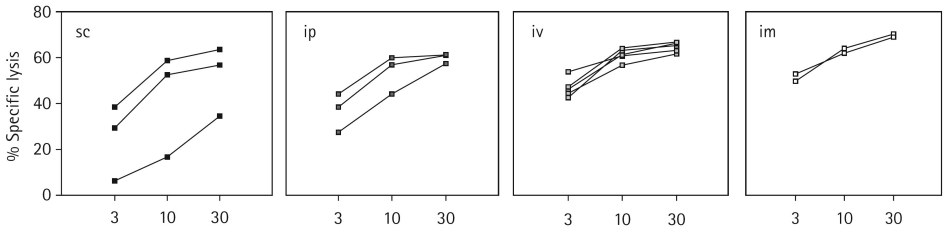

Figure 1. Influence of the route of immunization of SFV-enhE6,7 on bulk CTL activity. Mice were immunized s.c. (n=3), i.p. (n=3), i.v. (n=5) or i.m. (n=2) and boosted via the same route as the primary immunization, with $5x10^6$ SFV-enhE6,7 or PBS as control. Shown are the combined results of two separate experiments in which in the first immunization experiment 3 mice per group were immunized s.c., i.p. or i.v. and in the second experiment 2 mice per group were immunized i.v. or i.m.. CTL activity was determined one week after the booster immunization. After 7 days of *in vitro* restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. In the individual panels the levels of cytolysis at different effector to target ratios are shown. Less than 2% of lysis at an effector to target ratio of 30:1 was observed in mice injected with PBS (not shown).

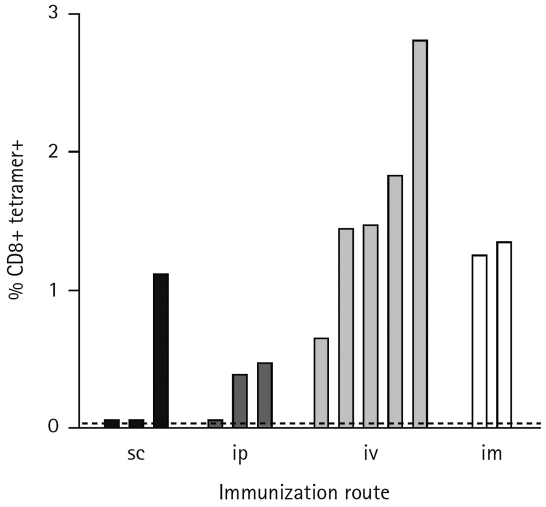

Figure 2. Influence of the route of immunization of SFV-enhE6,7 on pCTL frequency. Mice were immunized s.c. (n=3), i.p. (n=3), i.v. (n=5) or i.m. (n=2) and boosted via the same route as the primary immunization, with $5x10^6$ SFV-enhE6,7 or PBS as control. pCTL frequencies were determined from the same spleen populations that were analyzed for bulk CTL activity as shown in Figure 1. Spleen cells, directly upon isolation, were stained with a FITC-labeled monoclonal antibody against CD8 and a PE-labeled HPV16 $E7_{49-57}$ specific MHC class I tetramer and analyzed by flowcytometry. Shown are the percentages of $E7_{49-57}$ tetramer-positive $CD8^+$ cells of individual mice. The maximum background response in PBS injected mice is shown as a horizontal dotted line.

3A, circles) administered s.c. resulted in good CTL responses. However, 10^4 SFV-enhE6,7 (Figure 3A, triangles) immunized s.c. did not induce a detectable CTL response. On the other hand, 10^4 SFV-enhE6,7 (Figure 3B, triangles) administered i.v. resulted in a potent CTL response and even one of two mice generated CTLs upon immunization and boosting with as few as 10^3 SFV-enhE6,7 (Figure 3B, diamonds). I.v. immunization with 10^6 SFV-enhE6,7 resulted in comparable levels of cytolysis as 10^5 particles (not shown for clarity of the figure; also see figure 1).

Efficacy of SFV-enhE6,7 immunization with respect to memory CTL

To determine whether memory responses were induced upon SFV-enhE6,7 immunization, CTL responses were determined 1, 2 and 12 weeks after immunization with $2.5x10^6$ SFV-enhE6,7 injected i.v. (Figure 4). One week after a single immunization CTLs could be detected both by bulk CTL analysis of spleen cells and tetramer analysis of spleen and blood cells (Figure 4, data points A). The frequency of pCTLs in blood was 3-fold higher compared to the frequency in spleen.

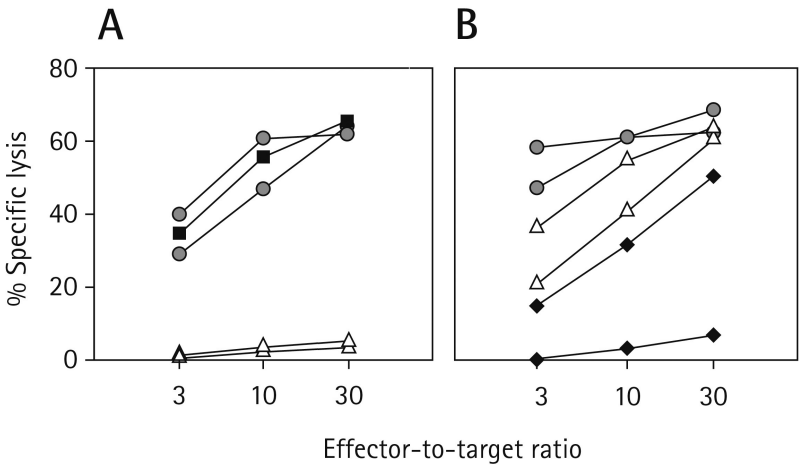

Figure 3. CTL activity induced upon immunization with an increasing dose of SFV-enhE6,7. Mice were immunized s.c. (panel A) or i.v. (panel B) and boosted with 10^6 SFV-enhE6,7 (squares), 10^5 SFV-enhE6,7 (circles), 10^4 SFV-enhE6,7 (triangle) or 10^3 SFV-enhE6,7 (diamonds). After 7 days *in vitro* restimulation the resulting effector cells were tested for cytolytic activity against 13-2 target cells in triplicate well assay. Shown are the levels of cytolysis at different effector to target ratios. Less than 2% of lysis at an effector to target ratio of 30:1 was observed in mice injected with PBS (not shown).

One week after a booster immunization the frequency in pCTLs in spleen increased 3-fold compared to a single immunization while also the pCTLs in blood increased (data points B). Two weeks after booster immunization, the pCTL frequencies both in spleen and blood decreased yet CTL activity as determined in bulk CTL assay remained at the same level as observed one week after immunization (data points C). Similarly, 12 weeks after booster immunizations the pCTLs were fully responsive to the in vitro restimulation employed in the bulk CTL assay, resulting in a high level of CTL activity at an effector to target ratio as low as 3:1 (data points D).

Influence of route of immunization on therapeutic efficacy of SFV-enhE6,7

We next determined whether the observed influence of the route of administration with respect to CTL induction is also reflected in the therapeutic effect of SFV-enhE6,7, using a murine HPV tumor model. Since it is unlikely that the i.p. route of administration will be applied in man, this route of administration was not analyzed further. In previous studies we demonstrated that s.c. immunization protects mice from a subsequently inoculated tumor and prevents tumor outgrowth when immunization is initiated early (2 days) after tumor inoculation[12]. To compare the different routes of administration we determined the therapeutic efficacy of SFV-enhE6,7 in the same model yet initiating the immunizations at later time-points. In

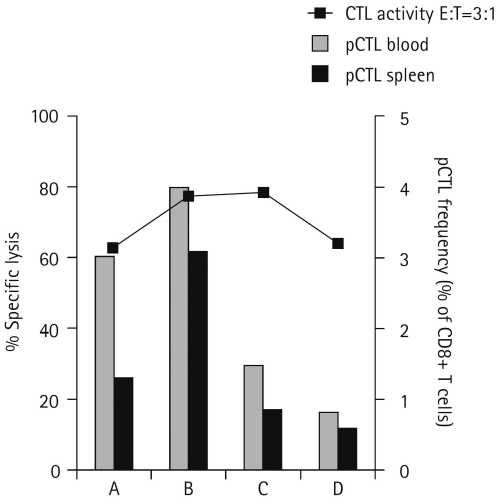

Figure 4. Efficacy of SFV-enhE6,7 immunization with respect to long-term pCTL frequency and CTL activity. Two mice were i.v. injected once with $2.5x10^6$ SFV-enhE6,7, one week after injection spleen and blood cells were analyzed for bulk CTL activity, as described in figure 1, and pCTL frequency using HPV16 $E7_{49-57}$ tetramers (data points A). Three other groups of two mice were injected twice with a two-week interval and spleen and blood cells were isolated either one week (data points B), two weeks (data points C) or twelve weeks (data points D) after the second injection. All material was analyzed in one CTL experiment and FACS analysis. Shown are the mean percentages of cytolysis of spleen cells at an effector to target ratio of 3:1 (squares). The bars represent the mean pCTL frequencies as measured in blood (gray bars) and spleen (black bars).

the first experiment, immunization was initiated seven days after s.c. inoculation of TC-1 cells in the neck of C57Bl/6 mice. As demonstrated in Figure 5A, all control mice (n=12) developed a tumor within 2 to 3 weeks after tumor inoculation. All mice immunized and boosted i.v. (n=14) with $5x10^6$ SFV-enhE6,7 eradicated the tumor and remained tumor-free for the next 3 months (Figure 5B). In the group of mice (n=21) immunized s.c. eleven of twenty-one mice eradicated the tumor, three developed a tumor at an early time point, comparable to control mice, and three mice developed a tumor at a much later time point, i.e. around week 10 (Figure 5C). Thus, the observed difference in CTL induction is also reflected in the therapeutic effect, i.e. i.v. immunization has a significantly superior therapeutic effect compared to the s.c. immunization.

To analyze whether i.m and i.v. immunizations are as effective in a therapeutic setting we first determined the lower limits of the therapeutic efficacy of i.v. immunization. For this purpose immunization was either initiated as late as 14 days or 17 days after tumor inoculation or mice were immunized with decreasing doses of SFV-enhE6,7. As shown in Figure 6B, initiation of immunization at day 7 results

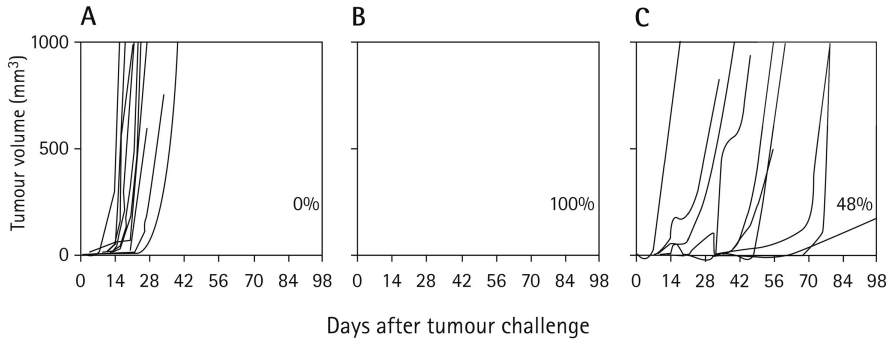

Figure 5. Growth and regression of HPV-transformed tumors upon i.v. and s.c. treatment with SFV-enhE6,7. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, mice were immunized and boosted i.v. (n=14; panel B) or s.c. (n=21; panel C) with $5x10^6$ SFV-enhE6,7 at days 7, 14 and 21 after tumor inoculation. As control, mice were injected i.v. with PBS (n=12, panel A) at days 7, 14 and 21. Tumor measurements were initiated 10-14 days after tumor challenge and subsequently measured twice weekly. Shown are the tumor volumes of individual mice. The percentages indicate the percentage of tumor-free mice at day 100 after tumor inoculation. At a tumor volume of approximately 1000 mm^3, the mice were sacrificed.

in eradication of tumor cells in all mice, confirming the results shown in Figure 5B. Initiation of immunization (Figure 6C) as late as day 14 resulted in a remarkable decrease in tumor mass in the 4 mice that developed an early tumor. In two mice the tumor mass decreased from a volume of 900 mm^3 and 750 mm^3 to 225 mm^3. In two mice tumors of 530 mm^3 and 115 mm^3 were completely eradicated. Ultimately, after ending the booster injections, tumor started to grow out again in three mice; however, four mice had cleared the tumor and remained tumor-free. A similar anti-tumor effect could be seen when immunization was initiated 17 days after tumor inoculation (Figure 6D). Unfortunately, three mice were killed before a possible immune response could be effective since the tumor reached the critical size of 1000 mm^3. One mouse had to be killed because its tumor, although very small, grew through the skin, which is also a criterion to kill the animal. In three mice again a very strong decrease in tumor volume was observed and ultimately three mice eradicated the tumor and remained tumor-free.

To determine the minimal effective dose, mice were immunized and boosted with decreasing doses of SFV-enhE6,7. As shown in Figure 7, immunization followed by two booster injections of $5x10^5$ SFV-enhE6,7 resulted in a complete therapeutic effect in that all mice eradicated the tumor and remained tumor-free for 10 weeks. Even upon immunization with as few as $5x10^4$ SFV-enhE6,7 six of seven mice eradicated the tumor.

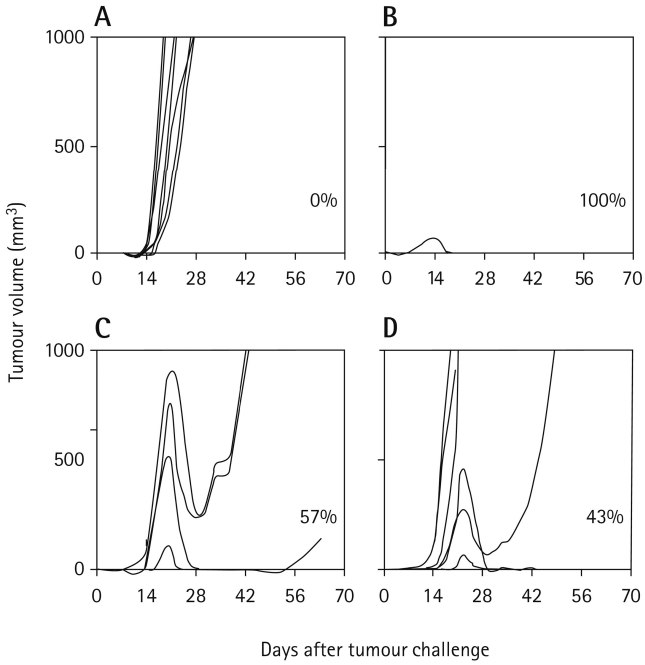

Figure 6. Efficacy of SFV-enhE6,7 immunization on regression of established tumors. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, mice were immunized and boosted i.v. with $5x10^6$ SFV-enhE6,7 at days 7, 14 and 21 (n=7; panel B), at days 14, 21, 28 (n=7; panel C) or at days 17, 24 and 31 (n=7; panel D) after tumor inoculation. As control, mice were injected i.v. with PBS (n= 10, panel A) at days 7, 14 and 21. Tumor measurements were initiated 10-14 days after tumor challenge and subsequently measured twice weekly. Given is the tumor volume of individual mice. The percentages indicate the percentage of tumor-free mice for each treatment at day 70 after tumor inoculation.

We next used two suboptimal treatment regimens to compare the efficacy of i.m. immunization with i.v. immunization. Mice were either immunized with $5x10^6$ SFV-enhE6,7 starting at day 14 after tumor inoculation or were immunized with 10^4 SFV-enhE6,7 starting on day 7. In the lower panels of Figure 8, tumor growth in mice immunized i.v. or i.m. at a late time-point is shown. Since in this experiment the tumor grew out relatively early (some tumors reaching volumes over 1000 mm^3 before an anti-tumor response could have been effective) we decided to let the tumors reach a size of 2000 mm^3 before killing the animal or kill the animal as soon as the tumor, after decreasing in size, started to increase again. Again, similar to the results shown in Figure 6, a tremendous decrease in large tumors was observed after i.v. immunization (Figure 8D). A similar response occurred upon i.m. immunization (figure 8C). In the end, in the i.m. group six of seven mice had to be killed because of tumor outgrowth and in four of seven mice immunized i.v. the tumor started to grow again. Upon immunization with

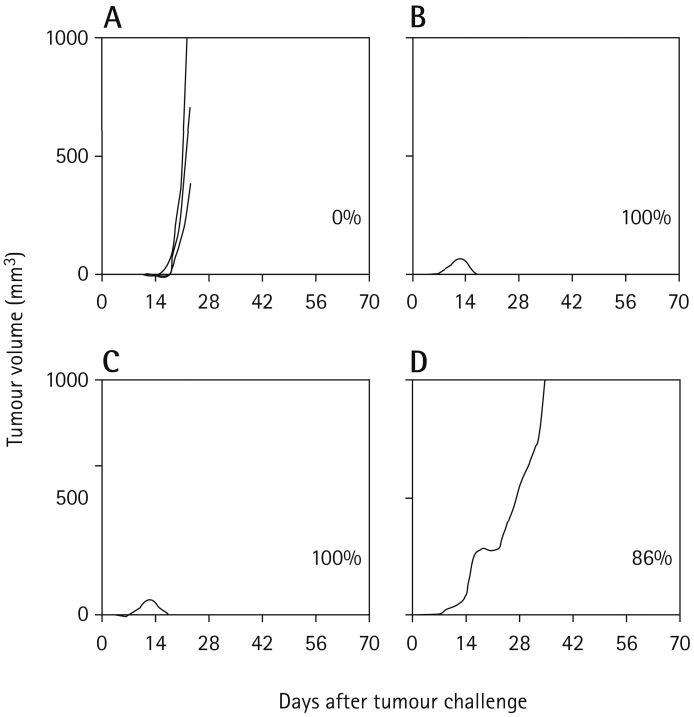

Figure 7. Growth and regression of HPV-transformed tumors upon i.v. immunization with a decreasing dose of SFV-enhE6,7. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, mice were immunized and boosted on days 7, 14 and 21 with $5x10^6$ SFV-enhE6,7 (n=7; panel B), $5x10^5$ SFV-enhE6,7 (n=7; panel C), $5x10^4$ SFV-enhE6,7 (n=7; panel D) or PBS (n=4; panel A), as control. Tumor measurements were initiated 10-14 days after tumor challenge and subsequently measured twice weekly. Given is the tumor volume of individual mice. The percentages indicate the percentage of tumor-free mice for each treatment at day 70 after tumor inoculation.

as few as 10^4 SFV-enhE6,7, five of seven mice immunized i.m. eradicated the tumor (Figure 8A) and in the i.v. group one of four eradicated the tumor (Figure 8B). Although the groups are relatively small it seems justified to claim that i.v. and i.m. administrations of SFV-enhE6,7 are equally effective in the induction of therapeutic anti-tumor responses in this murine tumor model.

Long-term anti-tumor efficacy of SFV-enhE6,7 immunization

We next questioned whether the CTL memory responses induced by SFV-enhE6,7 immunizations, as demonstrated in figure 4, suffice for an *in vivo* anti-tumor response. Therefore, several mice that eradicated the tumors in the previous therapeutic studies were re-challenged with tumor cells without an additional immunization. Of six mice that were immunized on days 7-14 and 21 after first

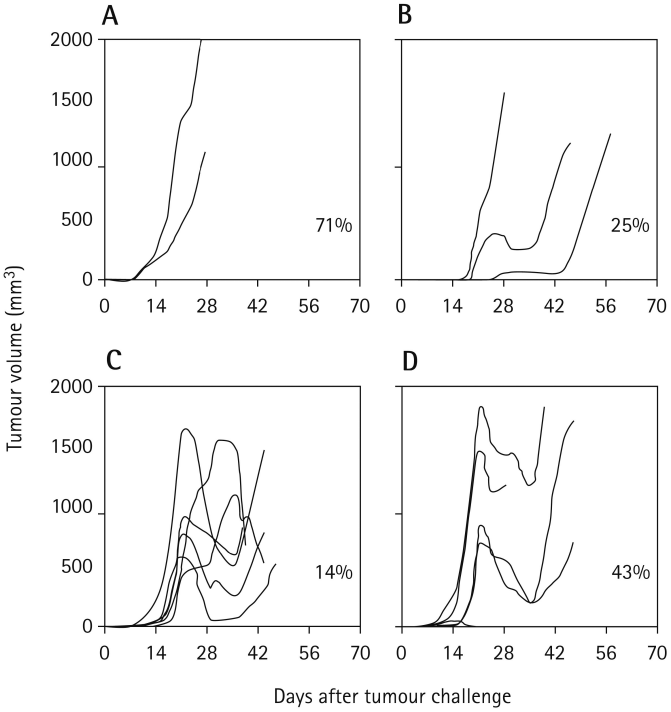

Figure 8. Comparison of anti-tumor effect induced upon i.v. or i.m. immunization with SFV-enhE6,7. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, two groups of mice were immunized and boosted on days 7, 14 and 21 with 10^4 SFV-enhE6,7 either i.m. (n=7; panel A) or i.v. (n=7; panel B). Two other groups were immunized with $5x10^6$ SFV-enhE6,7 on days 14,21 and 28 either i.m. (panel C) or i.v. (panel D).Tumor measurements were initiated 10-14 days after tumor challenge and subsequently measured twice weekly. Given is the tumor volume of individual mice. The percentages indicate the percentage of tumor-free mice for each treatment at day 70 after tumor inoculation.

tumor inoculation and had eradicated this tumor, four were able to eradicate the tumor of the second challenge 22 weeks after the first one. Tumor growth in the two mice that developed a tumor was strongly delayed, i.e. the tumors started to grow after 6 and 8 weeks, respectively. All mice included in this rechallenge experiment initially immunized at days 14-21-28 (n=3) or 17-23-30 (n=2) eradicated the tumors of the rechallenge (not shown).

DISCUSSION

In this study we demonstrate that robust immune- and anti-tumor responses against HPV16 induced tumors can be evoked by immunization with recombinant SFV expressing the oncoproteins E6 and E7 of HPV16. The magnitude of this

response is not only determined by the dose administered but also by the route of immunization. While we previously demonstrated that upon s.c. injection an antigen-specific CTL response is induced[12], we now show that the i.v. and i.m. routes of injection are far more effective than the s.c. and i.p. route of injection, both with respect to CTL induction as with respect to therapeutic anti-tumor responses. Despite the fact that the bulk CTL responses between the tested immunization routes do not differ significantly, tetramer analysis revealed that the i.v. and i.m. route of immunization result in significantly higher pCTL frequencies. A further analysis demonstrated that indeed over 100-fold fewer virus particles are needed when immunizing i.v. or i.m. as opposed to the s.c. route. Tumor treatment experiments show that i.v. and i.m. immunizations also result in superior anti-tumor responses compared to s.c. immunization, which can most likely be ascribed to the higher pCTL frequencies generated. The tumor treatment experiments, furthermore, clearly demonstrate the enormous potency of the vector. Exponentially growing tumors of approximately 500 mm^3 in size were seen to completely resolve and even some tumors as large as 1500 mm^3 decreased to one third of their size. Considering that a tumor nodule of 1000 mm^3 contains approximately 10^9 cells this implies that in the latter situation, i.e. a tumor decreasing 1000 mm^3 in volume, the CTLs generated, manage to kill 10^9 cells in one-week time. An other important aspect of our immunization approach is the observation that a long-term immune response, i.e. memory response, is induced as illustrated in figure 4 with respect to pCTL frequencies and in our observations that even 22 weeks after immunization mice can eradicate s.c. inoculated tumors[12].

Colmenero *et al.*[13] determined splenic CTL responses after injection of a high dose of SFV ($2x10^7$ particles) encoding the nucleoprotein of Influenza virus. In contrast to our conclusion, they conclude that injection of SFV via the i.v. and i.m. route is only slightly more effective than the s.c. route. The relatively small difference Colmenero *et al.* observed might be due to the high dose of SFV administered. The level of CTL activity induced upon i.v. or i.m. injection might already have been reached with 10 to 100-fold lower doses. In Colmenero's study the difference was not further evaluated nor was the effect of the immunization route on *in vivo* therapeutic responses determined. Also from our bulk CTL experiments (i.e. figure 1) one could conclude that the route of injection does not significantly influence CTL activity, yet tetramer analysis, dose-response studies and anti-tumor responses demonstrate that i.m. and i.v. injections are much more effective than the s.c. or i.p. route of injection.

Alphavirus–based immunization strategies are gaining more and more interest

because of their efficacy to evoke strong and long-lasting immune responses[18]. The recombinant SFV system activates both the innate and the adaptive immune system. Infection of cells with (recombinant) SFV results in dsRNA intermediates that are known for their immunopotentiating capacity[19]. dsRNAs can be recognized by innate immune receptors such as Toll-like receptor 3 and trigger production of Interferon type I, while, in addition, dsRNAs activate and mature DCs[20]. Uptake of apoptotic cells transfected with recombinant SFV will thus not only provide the specific antigen but also provide the required danger signal. Apart form their superiority, alphavirus vectors can safely be used for the expression of oncoproteins such as E6 and E7 since the viral RNA is not integrated in the host genome. Furthermore, since SFV is a rodent virus, humans in general do not carry neutralizing antibodies against the virus that may decrease the efficacy of the immunization. In addition to this, Berglund *et al.*[21] demonstrated that also upon immunization with SFV the immune responses against the SFV vector itself did not disable boost responses by subsequent immunizations with the same vector. Also with respect to the induction of anti-tumor responses against HPV-transformed tumors in animal models, immunization with recombinant alphaviruses has proven to be highly effective. Alphavirus-based immunizations result in high levels of pCTLs and CTL activity and most important in therapeutic anti-tumor responses as also demonstrated for recombinant Venezuelan Equine Encephalitis Virus (VVE) and Sindbis particles[22;23].

Expression, routing and processing will eventually determine the magnitude, duration and type of immune responses. Colmenero *et al.*[13] reported on the localization of viral RNA after injecting rSFV via different injection routes. They demonstrated that upon i.v. injection rSFV-RNA was distributed to a variety of different tissues whereas it was confined locally after i.m. and s.c. injections. Morris-Downes *et al.*[14] studied the distribution and persistence of i.m. injected rSFV in mice and chickens and observed that i.m. injected rSFV showed persistence at the injection site up to 7 days, transient detection in secondary lymphoid organs and no dissemination to distal sites. Using another alphavirus vector system, rVVE, Caley *et al.*[24] reported on the expression of the recombinant protein in popliteal lymph nodes upon s.c. injection in the footpath. Although not directly comparable, the difference in localization between SFV and VEE gene expression might be ascribed to the observation that while rSFV does not transfect DCs[9], rVEE does. Thus, upon rVEE injection DCs might become infected at the site of injection and subsequently migrate to local lymph nodes where they can evoke an immune response. SFV on the other hand, transfects a variety of cells such as fibroblast, primary human tumor cells, murine tumor cells lines[25;26], cardiovascular

cells and smooth muscle cells[27] but, as mentioned above, not DCs. The immune response evoked by SFV immunization occurs via cross-priming of antigen by DCs that have taken up apoptotic rSFV transfected cells[9]. Although these data may help us to predict or design the most optimal immunization schedule and route, for the evaluation of the immunization strategy, anti-tumor efficacy studies will remain essential.

The robust therapeutic immune responses elicited with SFV-enhE6,7, as presented in this study, positions recombinant Semliki Forest virus as a serious candidate for clinical evaluation for treatment of (pre)malignant cervical lesions.

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CHAPTER 5

Induction of Human Papilloma virus E6/E7-specific cytotoxic T lymphocyte activity in immune-tolerant, E6/E7-transgenic mice by immunization with a recombinant Semliki Forest virus vector

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Gene Ther. 2005 Sep;12(18):1410-4

ABSTRACT

Despite promising preclinical results of various therapeutic anticancer immunization strategies, these approaches may not be effective enough to eradicate tumors in cancer patients. While most animal models are based on fast-growing transplantable tumors, malignancies in for example cervical cancer patients in general develop much more slowly, which may lead to immune suppression and/or immune tolerance. As a consequence, the immunomodulating signal of any therapeutic immunization regimen should be sufficiently potent to overcome this immunocompromised condition. In previous studies, we demonstrated that an experimental vaccine against human papillomavirus (HPV)-induced cervical cancer, based on Semliki Forest virus (SFV), induces robust HPV-specific cellular immune responses in mice. Now we studied whether this strategy is potent enough to also prime a cellular immune response in immune tolerant HPV transgenic mice, in which CTL activity can not be induced using protein or DNA vaccines. We demonstrate that, depending on the route of immunization, SFV expressing HPV16 E6 and E7 indeed has the capacity to induce HPV16 E7-specific cytotoxic T cells in HPV-transgenic mice.

Cervical cancer is the third most common cancer among women worldwide. It is caused by infection with high-risk Human Papillomavirus (HPV), in particular types 16, 18, 31, 33 or 45. Indeed, in over 99% of all cervical carcinomas, DNA derived from these HPV types is detectable.[1] High-risk HPVs have the capacity to transform cervical epithelial cells by integrating the open reading frames encoding the viral early proteins E6 and E7 into the host cell genome. This integration may lead to constitutive overexpression of E6 and E7, mediating transformation of the cells to a malignant phenotype.[2] Since the continued production of E6 and E7 is required for the maintenance of the transformed phenotype, E6 and E7 in fact represent tumor-specific antigens in cervical carcinoma and premalignant HPV-transformed cells. As a consequence, E6 and E7 are potential targets for immunotherapeutic intervention strategies involving induction or stimulation of cytotoxic T lymphocyte (CTL) activity against HPV-transformed cells.[3]

It is likely that HPV-specific CTLs play an important role in the immunological control of tumor development after HPV infection.[3] Indeed, while the majority of sexually active women become infected with HPV, only a minority develops premalignant cervical lesions or cervical cancer.[4] In these patients, as expected, HPV-specific CTL activity is generally low,[5;6] suggesting that they have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens. It is possible that this tolerance arises peripherally at the level of the epithelial keratinocytes, the target cells for HPV. These keratinocytes lack co-stimulatory molecules such that presentation of viral antigens in the context of MHC class I molecules may result in the induction of anergy in relevant T cells, thus causing immunological tolerance. Recently, Steinman and Nussenzweig[7] proposed

that peripheral tolerance can also be induced by immature dendritic cells (DCs) which silence the T cell repertoire to self and environmental antigens captured in the steady state, i.e. in the absence of an acute infection or inflammation. This induction of tolerance by immature DCs would be mediated by peripheral T cell deletion or by the induction of regulatory T cells. Immunological tolerance for chronic or persistent viral pathogens, such as HPV, could develop in a similar manner. Irrespective of its precise origin, the 'putative' immunological tolerance for HPV antigens in patients with cervical carcinoma or premalignant cervical disease puts high demands on potential immunotherapeutic strategies, since such approaches need to break or overcome this tolerance in order to be effective.

In previous studies,[8-10] we have demonstrated that immunization of mice with recombinant Semliki Forest virus (SFV) expressing a fusion protein of HPV16 E6 and E7 (SFV-enhE6,7) not only induces strong and long-lasting CTL responses, but also effectively eradicates established tumors of HPV-transformed cells. In the present study, we used K10HPV16-E6/E7 transgenic (Tg) mice which constitutively express HPV16 E6 and E7 under the control of the keratin 10 promoter in the suprabasal layers of the epidermis. Upon immunization with E7 protein, these mice produce anti-E7 antibodies but no E7-specific CTLs.[11;12] The antibody response has been shown to be comparable to that of their non-transgenic littermates, demonstrating that E7-specific B cells as well as T helper cells are not deleted during development. By contrast, the CTL tolerance is extremely strong, since attempts to break it with protein or DNA vaccination have thus far been unsuccessful.[12;13] The inability of K10HPV16-E6/E7 Tg mice to mount an E7-specific CTL response is not due to a general CTL unresponsiveness, since these mice are capable of generating specific CTL activity against for example ovalbumin.[12] These results suggest that, similar to the HPV-transgenic murine model in which HPV16-E6/E7 is expressed from the K14 promotor, expression from the K10 promotor results in "split" tolerance, in which antigen-specific CTL responses are strongly suppressed but the antibody and T helper responses remain unaffected.[14] In the present study we demonstrate that immunization of K10HPV16-E6/E7 Tg mice with SFV-enhE6,7 does induce CTL activity. Although the mechanism and kinetics of tolerance in this mouse model certainly differ from that in the human clinical situation, these studies do demonstrate the potency of immunization with the SFV-enhE6,7 vector.

Spleen cells isolated from wild-type (Figure 1A) as well as Tg mice (Figure 1B), immunized with 10^7 i.u. SFV-enhE6,7, displayed a high level of cytolysis against C3 target cells (squares), while no significant HPV-specific CTL activity was observed in the SFV-LacZ and PBS control groups (closed and open circles, respectively). Although, as expected, the cytolysis in wild-type mice was higher

than in Tg mice, this result demonstrates that immunization of K10HPV16-E6/E7 Tg mice with SFV-enhE6,7 can induce E7-specific CTLs in these animals.

Next, using interferon-γ (IFN-γ) Elispot analysis,[15] we determined the number of HPV-specific IFN-γ-secreting T cells after 7 days of *in vitro* restimulation with 13-2 cells. As shown in Figure 2, immunization of wild-type (open circles) and Tg mice (solid circles) with SFV-enhE6,7 elicited T cells specific for $E7_{49-57}$. The background level of IFN-γ-secreting cells in the PBS and SFV-LacZ control groups is not E7-specific but due to the 7-day culture in the presence of IL-2. In these control groups the numbers of spots in wells stimulated with or without E7 protein

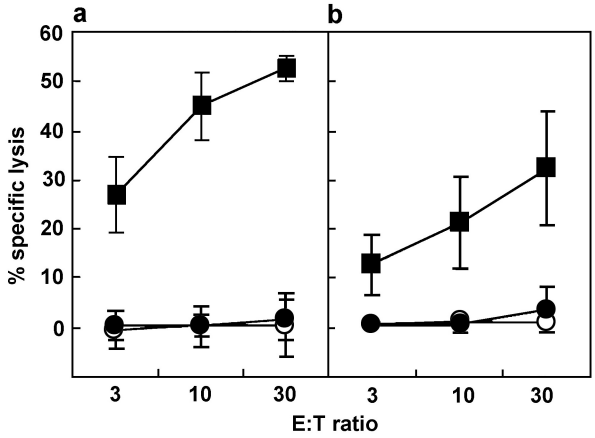

Figure 1. CTL activity induced in wild-type versus K10HPV16-E6E7 transgenic mice. Crossing of K10HPV16-E6/E7 Tg males and Tg or wild-type females produced F1 mice.[11] Expression of E6 and E7 was confirmed by PCR of DNA obtained from tail specimens. No difference in E6 and E7 expression was observed between homozygous and heterozygous F1 mice. In addition, no differences in CTL induction were observed between these mice (not shown). The production and purification of recombinant SFV-enhE6,7 and SFV-LacZ was performed as described previously.[8] (A) Wild-type mice immunized and boosted twice s.c. with 10^7 i.u. SFV-enhE6,7 (n=4, closed squares), 10^7 i.u. SFV-LacZ (n=2, closed circles) or PBS (n=4, open circles) (B) Tg mice immunized s.c. and boosted twice with 10^7 i.u. SFV-enhE6,7 (n=6, closed squares), 10^7 i.u. SFV-LacZ (n=2 closed circles) or PBS (n=3, open circles) as a control. Seven days after the last booster immunization spleen cells were isolated and co-cultured with irradiated (100Gy) TC-1 cells[28] (cell line generated from lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras) at a ratio of 25:1, in 25 cm^2 culture flasks, kept upright. After 5-6 days or 9-12 days of culture, cells were harvested and restimulated with irradiated naïve spleen cells (33Gy) and irradiated TC-1 cells (100Gy) at a ratio of 2:5:0.08 in 24-wells plates in the presence of 4 units of recombinant hIL2/ml. Five days after the first and/or second restimulation, cells were harvested and CTL activity was determined in a standard 4 h ^{51}Cr release assay, done in triplicate, using C3[29] (cell line derived from H-2^b embryonic cells transfected with a plasmid containing the complete HPV16 genome) or 13-2 cells[30] (cell line generated from H-2^b embryonic cells transfected with the E1-region of adenovirus type 5 in which an adenoviral epitope is replaced by the HPV16 E7 CTL epitope, AA 49-57) as targets. Similar levels of specific lysis were obtained using C3 or 13-2 cells; only the results obtained with the C3 cells will be shown.

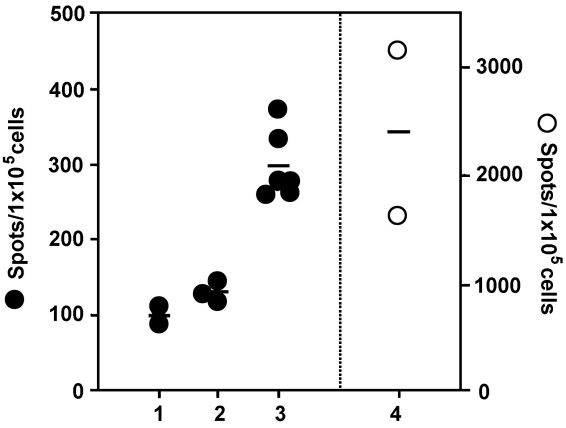

Figure 2. IFN-γ Elispot analysis of spleen cells from control and immunized Tg and wild-type mice. Tg mice (solid circles) were immunized and boosted twice with PBS s.c. (n=2; group 1), 10^7 i.u. SFV- LacZ s.c. (n=3; group 2), and 10^7 i.u. SFV- enhE6,7 s.c. (n=6; group 3) respectively. Wild-type mice (open circles) were immunized and boosted twice with 10^7 i.u. SFV-enhE6,7 s.c. (n=2; group 4). Seven days after the booster immunization spleen cells were isolated and cultured as described in the legend to figure 1. After a 7-day in vitro restimulation of spleen cells, the cells were harvested and cultured overnight on anti-IFNγ-coated ELISA plates in the presence of irradiated 13-2 cells and naïve spleen cells, in triplicate experiment. 13-2 cells were choosen as stimulator cells as these cells only express the HPV 16 E7 CTL epitope (AA 49-57) which is known to be recognized by H-2^b cells.The next day IFNγ-positive spots were developed as described previously analysis[15], and counted. Results are expressed as number of IFNγ-spots per 10^5 splenocytes. Each dot represents the median number of spots per 10^5 cells per mouse, the line indicates the group median.

were the same. Again similar to the bulk CTL analysis, the response in wild-type mice was higher than that in Tg mice. Assuming an equal doubling rate of pCTL from wild-type and Tg T cells during *in vitro* restimulation the Elispot results suggest that the pCTL frequency induced in wild-type mice is approximately 10-fold higher than that in Tg mice. The relatively low level of pCTLs induced in Tg mice can not be determined by direct *ex vivo* Elispot or tetramer analysis as these levels range around the detection limit of these methods (not shown). In contrast, pCTL levels in wild-type mice can be determined directly *ex vivo*, both by Elispot and tetramer analysis.[8]

Michel *et al.*[13] recently demonstrated that immunization of K10HPV16-E6/E7 Tg mice with VP22-$E7_{1-60}$ DNA was unable to overcome the E7-specific tolerance. Since the experimental conditions of the CTL assay used by these authors differed from the conditions in our experiments, we directly compared the efficacy of the DNA immunization with SFV-enhE6,7 immunization in our model. ŧŧŧŧA strong HPV-specific CTL activity was induced in wild-type mice upon immunization with

VP22-E7$_{1-60}$ DNA (Figure 3, open circles). In contrast, no HPV-specific CTL activity was observed in the Tg mice immunized with the DNA (Figure 3, closed circles), while immunization with SFV-enhE6,7 induced a significant HPV-specific CTL response in these mice (Figure 3, closed squares).

In wild-type mice, intravenous (i.v.) or i.m. injection of recombinant SFV results in higher levels of CTL activity compared to s.c. or i.p. injection.[10;16] To examine whether this also holds true in the K10HPV16-E6/E7 model, Tg mice were immunized via three different routes (s.c., i.m. or i.v.) with 10^7 i.u. SFV-enhE6,7. As shown in Figure 4, s.c. immunization induced a higher level of CTL activity compared to i.m. and i.v. immunization which is opposite to the effect of the injection route in wild-type mice.

The results presented in this report illustrate the exquisite potency of the SFV vector system as a means to induce CTL activity. While conventional DNA- and protein-based vaccines were unable to induce CTLs in K10HPV16-E6E7 Tg mice,[12;13] SFV-enhE6,7 did efficiently prime CTL activity in these mice.

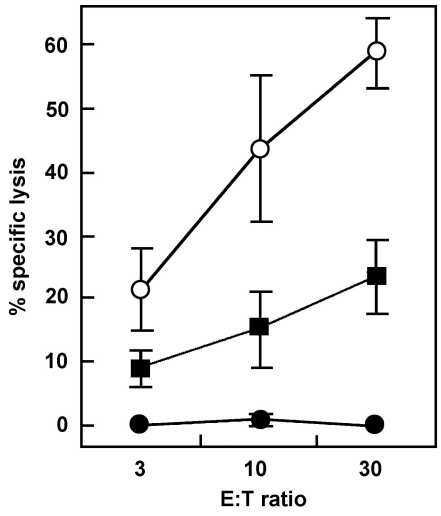

Figure 3. Induction of CTL activity upon SFV-enhE6,7 versus DNA-E7 immunization in wild-type and Tg mice. Tg (n=2; closed circles) and wild-type (n=3; open circles) C57Bl/6 mice were immunized intramuscularly (i.m.), in the tibialis anterior, with 100 μg VP22-E7$_{1-60}$ DNA.[13] The muscles of the mice were pretreated with 50 μl of 10 μM cardiotoxin 7 days prior to DNA injection. As a positive control, Tg mice were immunized s.c. and boosted twice with 10^7 i.u. SFV-enhE6,7 (n=2; closed squares). Ten days after i.m. injection of VP22-E7$_{1-60}$ DNA and 7 days after the last booster with SFV-enhE6,7, mice were sacrificed and CTL activity was determined as described above. μμShown are the levels of cytolysis at different effector-to-target ratios.

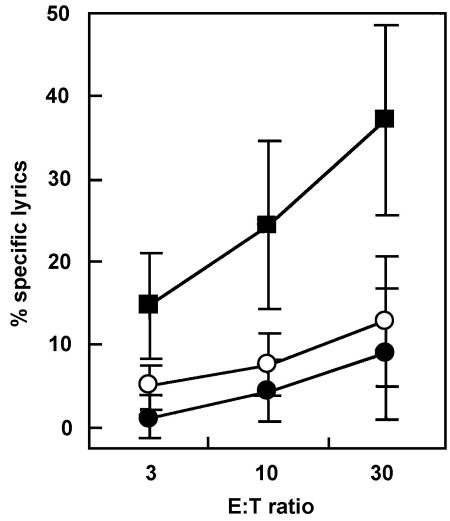

Figure 4. Effect of the route of immunization on the induction of CTL activity in Tg mice. Tg mice were injected s.c. (n=4, squares), i.m. (n=4, open circles) and i.v. (n=3, closed circles) with 10^7 i.u. SFV-enhE6,7. All mice were boosted twice s.c., i.m. and i.v. respectively. Seven days after the last booster immunization, CTL activity was determined in triplicate using C3 target cells. Shown are the levels of cytolysis at different effector-to-target ratios.

HPVs have evolved such that infected cells and (pre)malignant cells are not recognized effectively by the immune system. For example, expression of HPV E6 and E7 protein prevents the immunoregulatory effects of IFN-α- and IFN-β-mediated antiviral responses.[2;17] It is furthermore observed that women with cervical cancer develop poor levels of E7-specific CTLs in response to the developing tumor[5;6] or to an E7 vaccine.[19] This poor cellular immune response may be caused by the expression of E7 in epithelial cells lacking co-stimulation which may lead not only to immunosuppression but also to immunological tolerance. Scavenge of E7-expressing cells by Langerhans cells (LCs) or DCs without co-stimulation would thus prevent DC maturation. These immature DCs will transmit a tolerogenic rather than stimulatory signal to T cells. This and several other mechanisms of HPV-mediated immune escape have been recently reviewed by Tindle.[20] Considering these immune evasion strategies of HPV, Tindle proposes that immunization with any form of E7 will have to provide so-called "danger" signals[21] that turn immature, tolerizing DCs into mature, activating DCs. The recombinant SFV system would appear to meet these criteria in that both the innate and the adaptive immune systems are activated. Infection of cells with SFV results in the formation of dsRNA intermediates[22] that are known for their immunopotentiating capacity.[23] dsRNAs can be recognized by innate immune receptors, such as Toll-

like receptor 3, and trigger production of IFN type I. ln addition, dsRNAs induce activation and maturation of DCs.[24] Uptake of apoptotic cells transfected with recombinant SFV will thus not only provide the specific antigen but also provide the required danger signal.

The observation that in wild-type mice i.v. and i.m. immunization results in higher CTL responses than s.c. or i.p immunization, whereas in K10HPV16-E6E7 Tg mice the s.c. route seems superior, could be explained by the following observations. As described above, K10HPV16-E6E7 Tg mice express E6 and E7 in keratinocytes. Fausch *et al.*[25] recently demonstrated that human LCs, that reside in the epidermis of the skin or in epithelia of mucosal tissues, bind and internalize HPV virus-like particles but do not up-regulate markers nor initiate an HPV-specific immune response. Thus, LCs cells can be considered to be (co)responsible for the induction and maintenance of HPV tolerance. Breaking this tolerance could best be achieved by specifically targeting danger signals to tolerogenic LCs. As suggested above, immunization with recombinant SFV provides the danger signal that is needed to activate and mature LCs. Indeed, Johnston *et al.*[26] demonstrated that *in vivo* epidermal infection with SFV significantly increases the expression of MHC II, CD54 and CD80 on LCs. Thus, during an epidermal viral (SFV) infection, local LCs mature to a phenotype resembling that of lymphoid DCs. One could therefore speculate that s.c. immunization of the Tg mice with recombinant SFV used in this study might result in maturation and activation of skin LCs and disruption of immune tolerance. Upon i.v. or i.m. injection, SFV-infected cells are not likely to reach LCs.

Apart from the potent immune-stimulatory effect, infection of cells with replication-incompetent recombinant SFV is highly efficient and, in addition, it is safe. Infection of cells does not proceed beyond a single round and the infected cells die by apoptosis.[27] The transient nature of infection with recombinant SFV thus provides an important safety advantage when considering using SFV as a vaccine in humans.

In summary, we have demonstrated that immunization with recombinant SFV expressing a fusion protein of HPV16 E6 and E7 induces HPV-specific CTL activity in a very stringent HPV tolerogenic mouse model. Considering the effect of the route of immunization, we hypothesize that the ability of recombinant SFV to overcome immunological tolerance in this model is due to its high efficiency and mediated by the activation and maturation of tolerogenic LCs.

Acknowledgements

We thank Prof. L. Gissmann and Dr. N. Michel from the Deutsches Krebsforschungszentrum, Heidelberg, Germany for kindly providing us with founder transgenic K10HPV16-E6/E7 mice and VP22-$E7_{1-60}$ DNA, and Prof. J. E. Degener from our department for his support and encouragement.

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CHAPTER 6

Augmentation of alphavirus vector-induced human papilloma virus-specific immune and antitumor responses by co-expression of interleukin-12

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Manuscript in preparation

ABSTRACT

To enhance the therapeutic efficacy of an immunisation strategy against human papillomavirus-induced cervical cancer, based on the use of a recombinant Semliki Forest virus (SFV) vector system, we evaluated the adjuvant effect of interleukin-12 (IL12) co-expressed by a second SFV vector. We demonstrate that, depending on the dose and administration scheme, SFV-IL12 stimulates antigen-specific CTL responses elicited by a prime-boost immunisation regimen with recombinant SFV expressing a fusion protein of E6 and E7 of HPV16 (SFV-enhE6,7). In wild-type mice, SFV-enhE6,7-induced pCTL and CTL activity were enhanced by the addition of a low dose of SFV-IL12 to the s.c. prime immunisation with SFV-enhE6,7. Increasing doses of SFV-IL12 did not further stimulate the frequency of precursor CTLs and CTL activity. Rather the activity was reduced compared to the effect observed with the low dose of SFV-IL12. Addition of SFV-IL12 to the booster immunisation further reduced the efficacy of the SFV-enhE6,7 immunisation. A low-dose dose of SFV-IL12 added to the prime-immunisation improved the therapeutic anti-tumour efficacy of SFV-enhE6,7 immunisations in wild-type mice. In transgenic mice, tolerant for HPV E6 and E7, the addition of SFV-IL12 to the priming SFV-enhE6,7 immunisation also stimulated CTL responses. Our findings provide evidence for a specific enhancement of antigen-specific immune responses by SFV-IL12. However, our observations also show that prudence is called for when considering co-administration of SFV-IL12 to an immunisation strategy, as the enhancement of cell-mediated immune responses greatly depends on dosage and injection scheme.

INTRODUCTION

Cervical cancer is one of most common cancers among women worldwide.[1] It is caused by persistent infection with high-risk human papillomavirus (HPV), in particular types 16, 18, 31, 33 or 45. These high-risk HPVs have the capacity to transform cervical epithelial cells by integrating the open reading frames encoding the viral early proteins E6 and E7 into the host cell genome. This integration may lead to constitutive overexpression of these proteins, mediating transformation of the cells to a malignant phenotype.[2] Constitutive expression of E6 and E7 is a prerequisite for the maintenance of the transformed phenotype. This makes these proteins suitable targets for immunotherapeutic treatment of cervical cancer.[3]

Cell-mediated immune responses play an important role in the immunological control of HPV infections and early (pre)malignant lesions. In the majority of women, HPV-induced lesions regress spontaneously.[4] HPV-associated tumours occur more frequently in individuals treated with immunosuppressive drugs or patients infected with HIV[5-8] The present treatment of cervical neoplasia involves surgery (radical hysterectomy with pelvic lymphadenectomy) and/or radiotherapy combined with chemotherapy, depending on the stage of disease. As cervical cancer is a virus-induced disease and viral tumour antigens, which serve as targets for immunotherapy, have been identified, a tumour-specific vaccine could

further optimize treatment of patients with CIN and cervical cancer.[9] We have developed an immunotherapeutic vaccine based on a vector derived from the alphavirus Semliki Forest virus (SFV). Vectors based on alphaviruses are gaining increasing interest for their efficacy with respect to the induction of cellular immune responses.[10] In previous studies, we have demonstrated that immunisation of mice with recombinant SFV (rSFV) expressing a fusion protein of HPV16 E6 and E7 (SFV-enhE6,7) not only induces strong and long-lasting CTL responses but also effectively eradicates established tumours of HPV-transformed cells.[11-13]

Although these preclinical data with SFV-enhE6,7 are promising, prudence is called for when extrapolating these results to a clinical setting. HPVs have evolved such that infected cells and (pre)malignant cells are not recognized effectively by the immune system. For example, expression of HPV E6 and E7 protein prevents the immune-regulatory effects of IFN-α- and IFN-β-mediated antiviral responses.[14;15] It is furthermore observed that women with cervical cancer mount only poor levels of E7-specific CTLs in response to the developing tumour[16;17] or to an E7-vaccine[18], despite immunecompetence measured by other criteria. This poor cellular response may be caused by the expression of E7 in epithelial cells lacking co-stimulation, which may lead not only to immunosuppression but also to immunological tolerance.[19;20] A major challenge will therefore be to design immunotherapeutic regimens such that tumour-specific T cells are induced that are potent enough to turn the balance from immune tolerance towards immune activation.

To explore the potential ability of SFV-enhE6,7 to break immunological tolerance we previously performed an immunisation study in K10 HPV16 E6/E7 transgenic mice. These mice constitutively express HPV16 E6 and E7 under the control of the keratin 10 promoter in the suprabasal layers of the epidermis, making them tolerant for E6 and E7. Although immunisation and boosting with SFV-enhE6,7 did prime CTL activity in these transgenic mice, the levels of specific cytolysis induced upon SFV-enhE6,7 were much lower than those induced in wild-type mice.[21]

In the present study, we investigated whether co-administration of an immunostimulatory adjuvant to SFV-enhE6,7, can further enhance anti-tumour responses. A promising adjuvant for cancer vaccination strategies is IL12. Treatment with IL12 has been shown to have a marked anti-tumour and anti-metastatic activity in numerous animal models.[22] The ability of IL12 to induce antigen-specific immunity relies mainly on its ability to induce or augment Th1 and CTLs responses, and the induction of immune memory.[23-26] We evaluated whether the addition of SFV-IL12 to a recombinant SFV vaccine augments the induction of antigen-specific CTLs and anti-tumour responses. In wild-type mice

we determined if and at which dose and treatment schedule, SFV-IL12 could enhance the efficacy of an SFV-enhE6,7 immunisation. Next we evaluated this optimal immunisation regime in K10 HPV16 E6/E7 transgenic mice. Finally, the adjuvant activity of SFV-IL12 when administered in combination with SFV-enhE6,7 was analysed in tumour-treatment experiments.

RESULTS

Addition of SFV-IL12 to an SFV-enhE6,7 vaccine augments the induction of E6/E7 specific CTLs

In previous studies we have shown that in wild-type mice immunisation with SFV-enhE6,7 results in potent CTL responses as determined in bulk CTL assays. To enable the evaluation of the addition of SFV-IL12 to an immunisation with SFV-enhE6,7 in wild-type mice, the immunisation with SFV-enhE6,7 should be suboptimal, allowing the induction of higher levels of CTL- and anti-tumour activity. From previous studies we know that this can be achieved by immunising subcutaneously (s.c.) instead of the more effective intravenous (i.v.) or intramuscular (i.m.) route of immunisation and/or by lowering the dose of recombinant virus used for immunisation. [27]

To determine if addition of SFV-IL12 can enhance an antigen-specific immune response, increasing doses of SFV-IL12 (10^4, 10^5 and 10^6 infectious units) were added to a suboptimal s.c. immunisation with 10^6 I.U. of SFV-enhE6,7. SFV-IL12 was co-administered with the first immunisation (priming). Two weeks after the priming immunisation mice were boosted with SFV-enhE6,7 alone. One week after the booster the frequencies of HPV16 E6,7-specific precursor CTLs (pCTLs) were determined by tetramer analysis using MHC class I tetramers refolded with the $E7_{49\text{-}57}$ peptide (Figure 1), and CTL activity (Figure 2) was determined using a standard bulk CTL assay. Upon two s.c. immunisations with 10^6 I.U. of SFV-enhE6,7, as expected, no detectable pCTL frequencies were induced (Figure 1, bar B). However, the addition of SFV-IL12 evoked a strong enhancement in pCTLs, with a pCTL frequency up to 6% at a dose of 10^5 SFV-IL12 (Figure 1, bar D). This immunostimulating effect of SFV-IL12 was also reflected in CTL activity, as shown in Figure 2. Notebly, the CTL levels in mice immunised with the lower doses of SFV-IL12, i.e. 10^4 I.U. (Figure 2, panel C) and 10^5 I.U. (Figure 2, panel D), were higher than those in mice immunised with 10^6 SFV-IL12 (Figure 2, panel E).

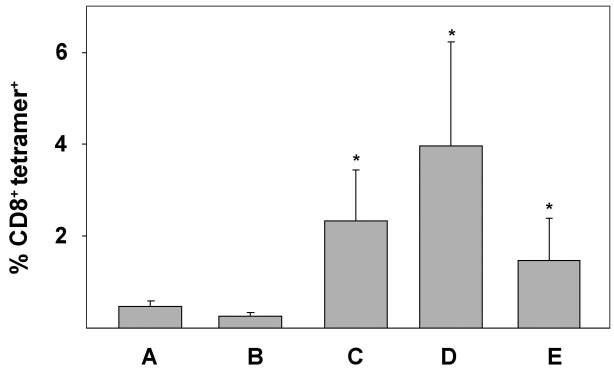

Figure 1. Effects of co-administration of SFV-IL12 with SFV-enhE6,7 on the induction of antigen-specific pCTLs. Mice were immunised and boosted s.c. with 10^6 SFV-enhE6,7, and increasing doses of SFV-IL12 were co-administered during the priming immunisation, i.e. 10^4 I.U. (n=5, C), 10^5 I.U. (n=5, D) and 10^6 I.U. (n=5, E). A group of 4 mice was immunised and boosted with SFV-enhE6,7 only (B), and 4 mice were immunised and boosted with PBS, as controls (A). pCTL frequencies were determined one week after the booster immunization. Spleen cells, directly upon isolation, were stained with a FITC-labelled monoclonal antibody against CD8 and a PE-labelled HPV16 $E7_{49-57}$ –specific MHC class I tetramer and analysed by flowcytometry. The average percentages (±SD) of $E7_{49-57}$ tetramer-positive CD8+ cells of the different groups are shown. Results of group C, D, and E were significantly different from group B ($p<0.05$)), as indicated with *.

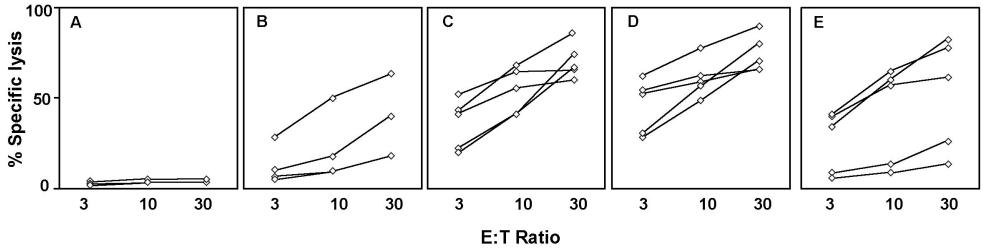

Figure 2. Effects of co-administration of SFV-IL12 with SFV-enhE6,7 on the induction of antigen-specific CTL activity. Mice were immunised and boosted as described in the legend to figure 1. CTL activity was determined after a 7-day *in vitro* restimulation. The resulting effector cells were tested for cytolytic activity against C3 target cells in triplicate well assay. In the individual panels A-E, corresponding to the bars depicted in Figure 1, the level of cytolysis at different effector-to target ratios is shown. Results in panel C and D were significantly different from panel B ($p<0.05$). The results of the group primed with 10^6 I.U. SFV-IL12 (panel E) were not significantly different from the SFV-enhE6,7 control (panel B).

Time-point of SFV-IL12 administration determines its efficacy

Next to the dose also the time-point of administration of IL12 has been described to be a critical factor for positive cell-mediated immunity effects.[28;29] To evaluate if the time-point of administration also influences the adjuvant activity of IL12 expressed by SFV, SFV-IL12 was added either to the priming SFV-enhE6,7

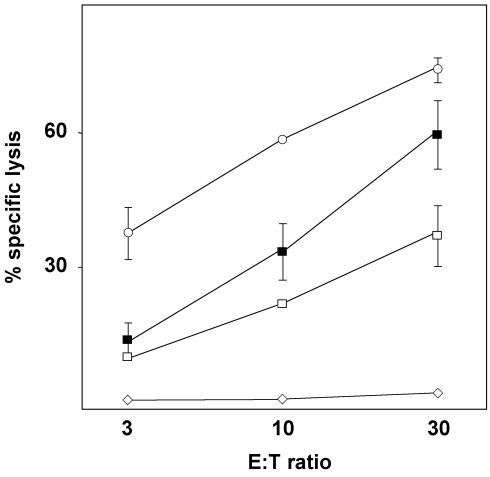

Figure 3. Effects of the timing of SFV-IL12 administration on the induction of antigen-specific CTLs.
Mice were immunised and boosted s.c. with SFV-enhE6,7 (10^6 I.U.) . SFV-IL12 (10^5 I.U.) was either co-administered at the priming (open circles), at the booster (open squares) or at both immunisations (not shown). As controls, mice were immunised and boosted with SFV-enhE6,7 only (closed squares) or injected with buffer only (closed diamands). One week after the booster, spleen cells were analysed for bulk CTL activity, as described in Figure 2. Shown are the resuls of a typical experiment. The average percentages (±SD) of cytolysis of spleen cells at different effector-to target ratios are depicted.

immunisation, to the booster immunisation or at both time-points. The addition of SFV-IL12 during the priming immunisation strongly enhanced CTL activity (Figure 3). In contrast, addition of SFV-IL12 to the booster reduced CTL activity. In mice in which SFV-IL12 was added both with the priming and the boosting immunisation with SFV-enhE6,7, the CTL activity strongly varied. In approximately half of these mice CTL activity was enhanced to the level also observed when adding SFV-IL12 to the prime only, while in the other half of the mice the response was reduced to the level also observed when adding SFV-IL12 to the booster immunisation (not shown). These findings indicate that in an rSFV prime-boost vaccine regimen, IL12 stimulates the immune response when co-expressed with antigen (i.e. E6,7) during the induction of generation of the initial primary immune response.

SFV-IL12 efficacy in HPV-transgenic mice

We previously demonstrated that in K10 HPV16 E6/E7 trangenic mice, tolerant for HPV16 E6E7, SFV-enhE6,7 immunisation can break this tolerance resulting in the induction of HPV-specific CTLs. Remarkably, in these transgenic mice tolerance can only be broken by s.c. immunisation with SFV-enhE6,7. The levels of specific cytolysis induced upon SFV-enhE6,7 were, however, much lower than those that can be induced in wild-type mice.[30] IL12 has been demonstrated to reverse antigen-induced tolerance and expand antigen-specific CTLs[31;32], by acting as a third signal along with TCR and costimulatory molecules.[33;34] To determine if SFV-IL12 could also enhance the induction of the specific CTL response in HPV-trangenic mice, K10 HPV16 E6/E7 trangenic mice were primed s.c. with 10^7 I.U. of

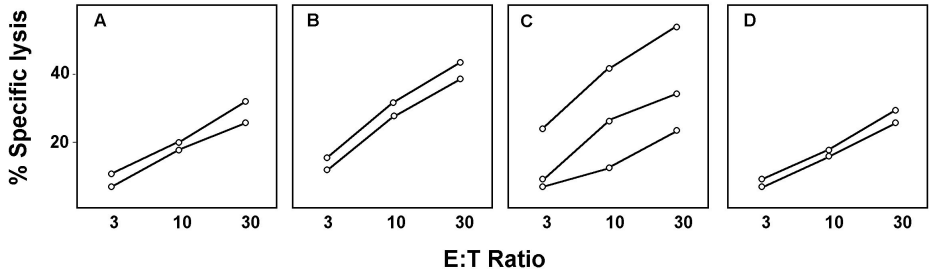

Figure 4. Effects of co-administration of SFV-IL12 with SFV-enhE6,7 on the induction of antigen-specific CTL activity in HPV-transgenic mice. K10 HPV16 E6/E7 trangenic mice were primed s.c. with 10^7 SFV-enhE6,7 together with increasing doses of SFV-IL12, 104 I.U. (panel B), 10^5 I.U. (panel C) or 10^6 I.U. (panel D) and boosted twice with 10^7 SFV-enhE6,7. A control group was immunised and boosted twice with 10^7 SFV-enhE6,7 only (panel A). Seven days after the last booster, spleen cells were isolated. After 12 days of *in vitro* restimulation, CTL activity was determined in triplicate using C3 target cells. Shown are the levels of cytolysis at different effector-to-target ratios. Less than 2% of lysis at an effector-to-target ratio of 30:1 was observed in mice injected with PBS (not shown).

SFV-enhE6,7 together with different amounts of SFV-IL12 particles and boosted twice with 10^7 SFV-enhE6,7. One week after the last booster injection, CTL activity was determined using bulk CTL assay. Compared to the immunisation with SFV-enhE6,7 only (Figure 4, panel A), addition of SFV-IL12 slightly improved the E6/E7-specific CTL response in these HPV-transgenic mice (panels B and C). The immune-potentiating effect of SFV-IL12 was dose-dependent yet not as strong as that seen in wild-type mice. Addition of 10^5 I.U. of SFV-IL12, the optimal adjuvant dose in wild-type mice, resulted in divergent CTL responses in HPV-transgenic mice. (Figure 4, panel C). The lowest dose of SFV-IL12 tested (10^4 I.U.) added to the priming immunisation of SFV-enhE6,7 slightly stimulated CTL activity in both wild-type and HPV-transgenic mice (Figure 4, panel B), while 10^6 I.U. SFV-IL12 did not enhance the immune response in HPV-transgenic mice (Figure 4, panel D). Thus, the enhancement in antigen-specific immune response elicited with a low dose of SFV-IL12, as observed in wild-type mice, also occurs in HPV-transgenic mice. However, the levels of specific cytolysis induced in the transgenic mice with the most optimal rSFV-vaccine regimen remained lower than those induced in wild-type mice.

Effect of co-administration of SFV-IL12 on *in vivo* anti-tumour response

The experiments described above revealed that SFV-IL12 enhances SFV-enhE6,7-induced specific CTL activity. To analyse whether this enhancement in CTL activity is also reflected in the therapeutic ability of the immune response to eradicate

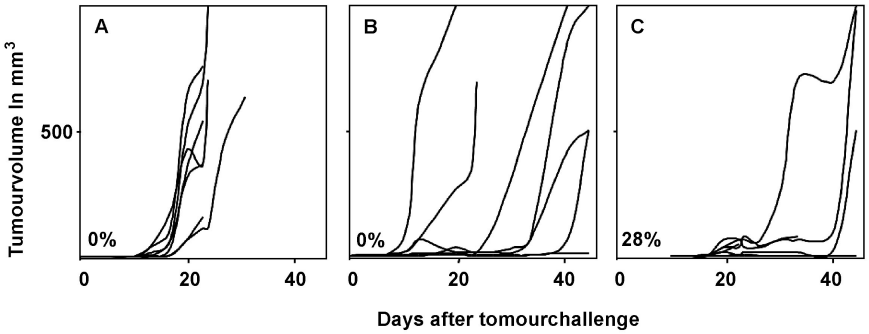

Figure 5. Effect of SFV-IL12 on the therapeutic anti-tumour efficacy of SFV-enhE6,7. Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently mice were immunised and boosted at day 7 and 14 with 10^6 SFV-enhE6,7 s.c., with co-administration of 10^5 SFV-IL12 at the priming (n=7, panel C) or without SFV-IL12 (n=7, panel B). As controls, mice were injected s.c. with PBS (n=7, panel A). Tumour measurements were initiated 10-14 days after tumour challenge and subsequently measured twice weekly. At a tumour of approximately 1000 mm^3, the mice were sacrificed. Shown is the tumour volume of individual mice. The percentages indicate the percentage of tumour-free mice at 6 weeks after tumour inoculation.

established tumours, a tumour-treatment experiment was performed. We previously have shown that optimal immunisation with SFV-enhE6,7 effectively eradicates established tumours of HPV-transformed cells in already 100% of the mice. To analyse if SFV-IL12 enhances the immune response in this tumour model, mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. Subsequently, mice were s.c. treated with a suboptimal dose of 10^6 SFV-enhE6,7 with or without 10^5 SFV-IL12 at day 7 and boosted with SFV-enhE6,7, only, at day 14. All control mice, injected s.c. with PBS, developed tumours within 14 days after tumour inoculation. (Figure 5, panel A) Although tumour outgrowth was significantly delayed, all mice immunised and boosted suboptimally with SFV-enhE6,7, developed tumours. (Figure 5, panel B) In contrast, 28% of the mice co-injected with SFV-IL12 at the priming were able to eradicate the tumour and remained tumour-free up to 45 days after tumour inoculation. Thus, the observed enhancement in specific CTL activity upon co-administration of SFV-IL12 is also reflected in the therapeutic effect.

We next determined the effect of co-injection of SFV-IL12 on tumour eradication in HPV16 E6E7 transgenic mice. The increase in CTL activity induced by SFV-IL12 when present during the priming immunisation, however, did not suffice to generate a sufficiently high anti-tumour response to kill the HPV-tumour cells *in vivo* in these tolerant mice (results not shown).

DISCUSSION

Interleukin-12 can target two processes in the control of tumour growth. The first process involves the formation of new blood vessels that are required for tumour nutrition, while the second process relates to the augmentation of immune responses directed against the tumour.[35-37] SFV encoding IL12 has sofar only been reported to effectively target the first process. In these studies, SFV-IL12 was administered intratumourally or peritumourally.[38-41] The local IL12 production, in this treatment setting, most likely results in an inhibition of tumour neovascularization through IFN-γ induction.[42] In the present study, we demonstrate that IL12 produced by SFV also targets the second anti-tumour process. Depending on the dose and administration scheme, SFV-IL12 enhances antigen-specific CTL responses elicited by a prime-boost immunisation with SFV-enhE6,7. In wild-type mice, SFV-enhE6,7-induced pCTL and CTL activity were enhanced by the addition of a low dose of SFV-IL12 (10^4 and 10^5 I.U.) to the priming s.c. immunisation with SFV-enhE6,7. Increasing the dose of SFV-IL12 (10^6 I.U.), did not further stimulate the frequency of precursor CTLs and CTL activity. Rather the activity was decreased compared to the effect observed with 10^4 and 10^5 I.U. of SFV-IL12. The addition of SFV-IL12 to the booster immunisation even suppressed the efficacy of the SFV-enhE6,7 immunisation alone. In transgenic mice, tolerant for HPV E6 and E7, the addition of SFV-IL12 to the priming immunisation with SFV-enhE6,7 enhanced CTL activity. The adjuvant activity of SFV-IL12 also improved the therapeutic anti-tumour efficacy of SFV-enhE6,7 immunisations in wild-type mice. However, such an effect was not achieved in K10 HPV16 E6/E7 transgenic mice.

As mentioned above, recombinant IL12 has been shown to have an anti-tumour and anti-metastatic effect in animal models.[22] IFN-γ is the principal mediator of the anti-tumour effects of IL12, through direct toxicity and activation of potent anti-angiogenic mechanisms via interferon-inducible protein-10 and monokine activity. [43-45] The major objection against IL12 for anti-tumour therapy is its toxicity. Dosages that elicit anti-tumour effects have severe side-effects. The dosages that elicit these anti-tumour responses far exceed the dose of IL12 produced upon the injection of SFV-IL12. For therapeutic effects, s.c. dosing schedules of for example 50-300 ng/kg twice weekly for a period of 24 weeks have been evalutated in patients. Colmenero and coworkers demonstrated that *in vitro* 6 ng of IL12 can be produced by the infection of 10^6 cells with SFV-IL12 at an MOI of 10.[46] Assuming that *in vivo* and *in vitro* cells produce similar amounts of IL12, this would imply that a maximum of 0.6 ng of IL12 is produced when injecting 10^5 IU of SFV-IL12 in a mouse (i.e. appr. 30 ng/kg). With a single injection of SFV-IL12 one would

therefore not expect any significant side effects. Indeed, in our experiments we did not observe any side effects.

IL12 as a recombinant protein has also been used as an adjuvant for the potentiation of antigen-specific CTL responses and increased anti-tumour therapeutic efficacy in several vaccination strategies in both mice and humans.[47] Co-administration of tumour antigens and IL12 can provide an environment with inflammatory danger signals that is required to activate antigen-presenting dendritic cells (DC) and may thereby prevent or revert tolerance to tumour-associated antigens.[48;49] The ability of IL12 to induce antigen-specific immunity relies mainly on its ability to induce or augment Th1 and CTL responses, and the induction of immune memory.[50-53] Apart from these studies in which IL12 is administered as a recombinant protein, several studies report on the effectiveness of IL12 as an adjuvant when expressed by a viral vector.[54;55]

Colmonero *et al.* showed that in a murine P815 s.c. tumour model, peri-tumoural or intra-tumoural co-delivery of SFV encoding the tumour antigen P1A and IL12 did not significantly improve the anti-tumour therapeutic effect obtained when the vectors were used individually.[56] The authors furthermore demonstrated that when both vectors were injected s.c. in the contralateral side of the tumour no anti-tumour effect was observed. The lack of an adjuvant effect in Colmenero's studies as well as the observation that contralateral s.c. injections did not evoke an immune response differ from our results. This difference in outcome can possibly be explained by our observation that the adjuvant effect of SFV-IL12 is dose- and schedule-dependent. In Colmenero's studies, 10^6 I.U. of SFV-IL12 were administered, a dosage that in our study inhibited CTL activity. The authors furthermore injected mice four times with SFV-IL12 and SFV-E-P1A. Although we did not evaluate the effects of four injections, we did observe that the addition of SFV-IL12 to the booster injection with SFV-enhE6,7 suppressed CTL activity, while the addition of SFV-IL12 to both the priming and the boosting immunisation in half of the mice augmented CTL responses but in the other half suppressed the CTL response. Thus while the effect Colmenero *et al.* observed when injecting SFV-IL12 peritumoural or intratumoural may be ascribed to an inhibition of tumour neovascularization through IFN-γ induction[57], the effect we observe with SFV-IL12 is most likely due to an immune-adjuvant effect of IL12. It should however be noted that the mouse strains used for both studies differ. Colmenero *et al.* used DBA/2 and Balb/C mice with a H2-d background that are more prone to develop Th2-type responses, while the C57Bl/6 mice used in our studies are more prone to a Th1-type of response. Further studies are needed to evaluate if this difference in genetic background influences the effects of SFV-IL12.

In the present study, we observed that the addition of SFV-IL12 to the priming immunisation with SFV-enhE6,7 enhances CTL responses, while it hampers the responses when given at the booster immunisation. These results are in line with a study by Gherardi *et al.*[58] These authors demonstrated that, in a heterologous DNA prime and vaccinia virus boost vaccine regimen, an optimal cellular immune response was induced when IL12 was delivered during the priming immunisation followed by a booster with the antigen alone. By contrast, Seaman *et al.*[59] demonstrated that plasmid IL12 delivery on day 10 post-immunisation resulted in a strong expansion of the number of CD8+ T cells specific for the antigen used in the study (GP120). This increase was not observed when the plasmid IL12 was administered on the day of the immunisation. However, a further analysis indicated that the expanded cells were primarily effector memory rather than central memory T cells. Central memory T cells have been shown to have a greater capacity to expand *in vivo* following an infection and are able to confer better protection than effector memory T cells. The data of these authors suggest that activation of CD8+ T cells during the peak phase of the immune response (day 10) drives the terminal differentiation into effector memory CTL. Further studies will have to demonstrate if the enhancement of the CTL response observed in our studies can be ascribed to the fact that administration of IL12 during the priming of naïve T cells drives the response to a central memory phenotype.

In conclusion, our results demonstrate that SFV-IL12 can enhance but can also hamper antigen-specific CTLs and anti-tumour responses elicited by a prime-boost immunisation with SFV-enhE6,7. In HPV-transgenic mice, tolerant for HPV16 E6E7 this enhanced efficacy slightly increases CTL activity yet does not suffice to induce anti-tumour responses. As however the tolerance in this transgenic mouse model is very stringent, the results in the wild-type mice suggest that one could consider to include SFV-IL12 as an adjuvant in SFV-based immunisations. The dose-response effect of SFV-IL12 requires that specific attention should be given to determine the optimal adjuvant dose. More is not always better.

MATERIALS AND METHODS

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (# CCL-10). The cells were grown in GMEM (Invitrogen, Breda, The Netherlands) containing 5% fetal calf serum. C3 cells, 13-2 cells, and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). The C3 cell line was derived from C57Bl/6 (H-2^b) embryonic cells

transfected with a plasmid containing the complete HPV16 genome.[60] The 13-2 cell line was generated from C57Bl/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF).[61] The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras.[62] C3, 13-2 and TC-1 cells were grown in IMDM with Glutamax-I (Invitrogen) supplemented with 10% fetal calf serum and penicillin and streptomycin (Invitrogen; 100 U/ml and 100 μg/ml, respectively). TC-1 medium was furthermore supplemented with non-essential amino acids (Invitrogen; 100-fold dilution), sodium pyruvate (Life Technologies; 1 mM) and Geneticin G418 Sulphate (Roche, Germany; 5mg/ml).

Mice

For wild-type mice, specific-pathogen-free female C57Bl/6 mice (Harlan CPB, Zeist, The Netherlands) were used. Founder transgenic mice, K10 HPV16 E6/E7, were kindly provided by Prof. L. Gissmann (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Crossing of K10HPV16-E6/E7 transgenic males with K10HPV16-E6/E7 transgenic or wild-type females produced F1 mice.[63] Expression of E6 and E7 was confirmed by PCR of DNA obtained from tail specimens. No difference in E6 and E7 expression was observed between homozygous and heterozygous F1 mice. In addition, no differences in CTL induction were observed between these mice (not shown). Both the wild-type mice and the HPV-transgenic mice were between 6 and 10 weeks of age at the start of the immunisation protocols.

Production, purification, and titer determination of rSFV

The production, purification, and titer determination of recombinant SFV-enhE6,7 and SFV-IL12 was performed as described previously.[64;65] In short, pSFV3-eE6,7 and pSFV-IL12 were produced using pSFV-Helper 2 and quantified using BHK 21 cells. pSFV3-eE6,7 encodes an enhanced expression of a fusion product of E6 and E7 of HPV type 16. For the construction of recombinant SFV-IL12, the genes encoding the p40 and p35 subunit proteins of murine IL12 were inserted individually downstream of the 26S promoter in the SFV expression vector pSFV4.2.

Immunisations

For CTL analysis, wild-type mice were immunised with a suboptimal s.c. immunisation with 10^6 I.U. of SFV-enhE6,7, followed by one booster immunization with a two-week interval. Varying doses of SFV-IL12 (10^4, 10^5 and 10^6 I.U.) were added either to the priming SFV-enhE6,7 immunisation, to the booster immunisation or at both

time-points. K10 HPV16 E6/E7 trangenic mice were primed s.c. with 10^7 I.U. of SFV-enhE6,7 together with different amounts of SFV-IL12 particles (10^4, 10^5 and 10^6 I.U.) and boosted twice with 10^7 SFV-enhE6,7. As negative controls, both wild type and HPV-transgenic mice were injected with PBS.

CTL assay

Seven to ten days after immunisation, spleen cells were isolated and cocultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm^2 culture flasks, placed upright. In experiments using wild-type mice one-week after in vitro restimulation, cells were harvested and a CTL assay was performed by a standard 4-hr ^{51}Cr release assay in triplicate. In experiments using HPV-transgenic mice, after 5-6 days and 9-12 days of culture, cells were harvested and restimulated with irradiated naïve spleen cells (33Gy) and irradiated TC-1 cells (100Gy) at a ratio of 2:5:0.08 in 24-wells plates in the presence of 4 units of recombinant hIL2/ml (Strathmann Biotech, Hamburg, Germany). Five days after the first and second restimulation, cells were harvested and CTL activity was determined in a standard 4 h ^{51}Cr release assay. Target cells (13-2 cells and C3 cells) were labeled for 1 h with 3.7 MBq $^{51}Cr/10^6$ cells in 100 μl medium (MP Biomedicals, Inc., Irvine, CA, USA). The mean percentage of specific ^{51}Cr-release was calculated according to the formula: % specific release = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] cpm x 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the mean. Similar levels of specific lysis were obtained using C3 or 13-2 cells; only the results obtained with the C3 cells are shown.

MHC class I tetramer staining and FACS analysis

To analyze the number of $CD8^+$ T cells specific for the HPV 16 $E7_{49-57}$ peptide RAHYNIVTF we used K^b-RAHYNIVTF tetramers produced in the laboratory of Dr. Ton Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Spleen cells were washed with FACS buffer (PBS containing 0,5% BSA and 0,02% sodium azide) and stained with FITC-conjugated anti-CD8a (Pharmingen, San Diego, CA, USA) together with PE-conjugated K^b-RAHYNIVTF tetramers for 20 minutes at 4°C. Spleen cells were washed three times and analyzed by flow cytometry (FACSCalibur, Beckton Dickinson, Breda, The Netherlands). Live cells were selected based on propidium iodide exclusion.

Tumour treatment experiments

Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Invitrogen). Subsequently, wild-type mice were treated s.c. with 10^6 particles of SFV-enhE6,7 with or without 10^5 SFV-IL12 at day 7, after tumour inoculation and boosted at day 14, with 10^6 particles of SFV-enhE6,7 only. HPV-transgenic mice were treated s.c. with 10^7 particles of SFV-enhE6,7 with or without 10^5 SFV-IL12 at day 2 and boosted at day 7 and 14 with 10^6 particles of SFV-enhE6,7 only (results not shown). As negative controls, both wild type and HPV-transgenic mice were injected with PBS after tumour inoculation. Tumour measurements were always performed blindly by the same technician. At a tumour volume of approximately 1000 mm^3, the mice were sacrificed.

Statistical analysis

Data depicted in Figures 1 and 2 were statistically analysed using the Mann-Whitney *U*-test.

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CHAPTER 7

A comparative study on the immunotherapeutic efficacy of recombinant Semliki Forest virus and adenovirus vector systems in a murine model for cervical cancer

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Gene Ther. 2007 Dec;14(24):1695-704

ABSTRACT

Currently, various therapeutic strategies are being explored as a potential means to immunize against metastatic malignant cells or even primary tumours. Using recombinant viral vectors systems or protein-based immunization approaches, we are developing immunotherapeutic strategies against cervical cancer or premalignant cervical disease, as induced by high-risk type human papillomaviruses (HPVs). We previously demonstrated that immunization of mice with recombinant replication-defective Semliki Forest virus (rSFV) encoding a fusion protein of HPV16 E6 and E7 (SFV-enhE6,7) induces strong CTL activity and eradication of established HPV-transformed tumours. In the present study, we compared the anti-tumour efficacy of SFV-enhE6,7 with that of a recombinant adenovirus (rAd) type 5 vector, expressing the same antigen construct (Ad-enhE6,7). Prime-boosting with SFV-enhE6,7 resulted in higher precursor CTL (pCTL) frequencies and CTL activity compared to prime-boosting with Ad-enhE6,7 and also in murine tumour treatment experiments SFV-enhE6,7 was more effective than Ad-enhE6,7. To elicit a therapeutic effect with Ad-enhE6,7, 100/1000-fold higher doses were needed compared to SFV-enhE6,7. *In vivo* T-cell depletion experiments demonstrated that these differences could not be explained by the induction of a different type of effector cells, since $CD8^+$ T-cells were the main effector cells involved in the protection against tumour growth in both rSFV- and rAd-immunized mice. Also comparable amounts of *in vivo* transgene expression were found upon immunization with rSFV and rAd encoding the reportor gene luciferase. However, anti-vector responses induced by a single injection with rAd resulted in a more than three log decrease in luciferase expression after a second injection of rAd. With rSFV, transgene expression was inhibited by only one to two orders of magnitude in pre-injected mice. As an antigen-specific booster immunization strongly increases the level of the CTL response and is essential for efficient induction of immunological memory, it is likely that (part of) the difference in efficacy between rSFV and rAd type 5 can be ascribed to a diminished efficacy of the booster immunization in the case of rAd due to anti-vector antibody responses.

INTRODUCTION

We are developing immunotherapeutic strategies against cervical cancer or premalignant cervical disease using replication-defective recombinant viral vector systems [1-5] or protein-based immunization approaches.[6] Cervical cancer is the second most common type of cancer among women worldwide, and the first malignancy recognized by the WHO to be 100% attributable to infection with a virus, specifically a high-risk type human papillomavirus (HPV). [7;8] Malignant cell transformation by HPVs involves integration of part of the viral genome into the chromosomal DNA of susceptible cervical epithelial cells. Subsequent constitutive expression of the E6 and E7 early viral genes results in inhibition of the tumour suppressor proteins p53 and pRb, respectively, and thus in a loss of cell cycle control.[9;10] Since the sustained expression of E6 and/or E7 is a prerequisite for maintenance of the transformed cellular phenotype, these proteins in fact

represent tumour-specific antigens and thus are potentially suitable targets for immunotherapeutic strategies against cervical cancer or pre-malignant cervical disease.[11] Therapeutic immunization against tumour cells requires the induction of cytotoxic T lymphocytes (CTL) that can specifically recognize and lyse tumour cells. For the differentiation, expansion and memory induction of tumour-specific CTLs, T-helper cells (Th cells) and properly activated antigen-presenting cells (APC), dendritic cells (DCs) in particular, are essential.

Preclinical evaluation of a recombinant replication-defective Semliki Forest virus (SFV) vector, expressing a fusion proteins of HPV type 16 E6 and E7, has demonstrated the ability of this system to induce strong E6/E7-specific cytotoxic T lymphocyte (CTL) activity in normal [1;3] and immune-tolerant E6/E7-transgenic [4] mice and to eradicate pre-established, subcutaneously implanted, HPV-transformed tumours in a murine tumour model.[2] However, despite the apparent potential of this approach, clinical evaluation of an SFV-based therapeutic immunization strategy is hampered by the fact that there is no prior human clinical experience with this vector system which would facilitate regulatory procedures.

A recombinant viral vector that has been evaluated extensively in human clinical studies is the adenovirus system.[12-14] Like recombinant SFV-based vector systems, recombinant adenoviruses (rAd) are attractive vaccine candidates, harbouring several advantageous features. rAd are easy to construct and propagate, they are replication-incompetent and can transduce a wide range of dividing and non-dividing cells, including antigen-presenting cells (APCs), and give high levels of transgene expression.[15;16] The only major limitation in the clinical application of rAd is the prevalence of pre-existing or inducible anti-viral immunity.[17;18]

In the present study, with the goal of rapidly bringing a therapeutic genetic immunization approach against (pre)malignant cervical disease to the clinic, we evaluated a recombinant replication-defective adenovirus vector expressing HPV16 E6/E7 antigen, in our murine model system and compared its efficacy with that of SFV-mediated immunization in terms of induction of CTL activity and anti-tumour responses.

RESULTS

Efficacy of Ad-enhE6,7 compared to SFV-enhE6,7 with respect to the induction of HPV-specific CTLs

To compare the efficacies of rAd and rSFV as vectors for the induction of anti-tumour activity, rAd and rSFV was generated encoding the same HPV16 E6,7 construct. This construct encodes the E6 and E7 protein of HPV16 as a fusionprotein.[1] Next,

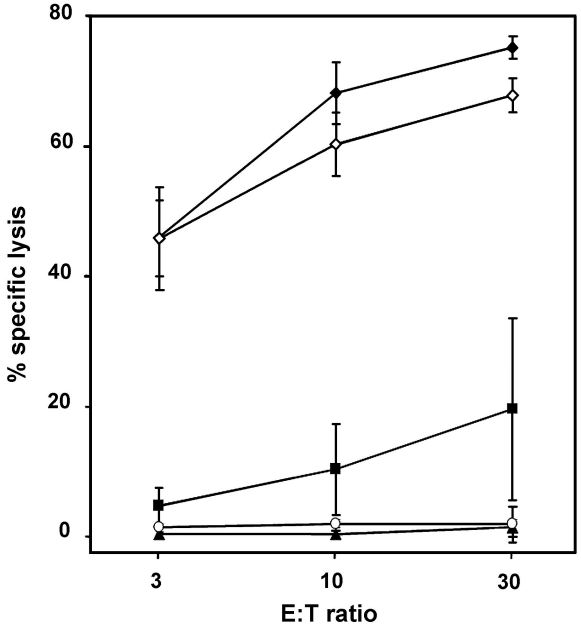

Figure 1. Efficacy of Ad-enhE6,7 compared to SFV-enhE6,7 with respect to the induction of HPV-specific CTLs

CTL-activity induced upon immunization with SFV-enhE6,7 particles versus Ad-enhE6,7 particles. Mice were immunized and boosted, with $5x10^6$ SFV-enhE6,7 i.v. (n=7, closed diamonds), with $5x10^6$ SFV-enhE6,7 i.m. (n=7, open diamonds), $5x10^8$ Ad-enhE6,7 i.m. (n=7, closed squares) or with $1x10^9$ Ad-Luc i.m. (n=3, closed triangles) or PBS (n=3, open circles) as controls. CTL activity was determined 1 week after the booster immunization. After 7 days of *in vitro* restimulation, the resulting effector cells were tested for cytolytic activity against C3 target cells in triplicate well assay. The levels of cytolysis at different effector-to-target ratios are shown. Control immunizations with $5x10^6$ SFV-LacZ did not induce CTL activity (not shown).[1,3] Shown are the results of a typical experiment repeated three times. Both the i.v. and i.m. SFV-enhE6,7 immunization groups and i.m. Ad-enhE6,7 immunization group were significantly different compared to the PBS control group (resp. p=0.03; p=0.03 and p=0.006). Both routes of SFV-enhE6,7 immunizations were significantly different compared to Ad-enhE6,7 (P=0.006)

Ad- enhE6,7 and SFV-enhE6,7 particles were compared in immunization and *in vivo* tumour treatment experiments. pCTL frequencies and CTL activity were determined one week after the last (booster) immunization by the direct staining of spleen cells with MHC class I tetramers refolded with HPV16 $E7_{49-57}$ peptide and a standard bulk CTL assay, respectively.

As shown in Figure 1, two intramuscular (i.m.) or intravenous (i.v.) immunizations with $5x10^6$ SFV-enhE6,7 resulted in a significantly higher CTL activity than two i.m. immunizations with a 100-fold higher dose of Ad-enhE6,7. As a control, mice

were injected twice with a two-fold higher dose of rAd encoding an irrelevant gene (Ad-Luc) or with SFV-LacZ (not shown) which did not evoke any CTL activity. Similar levels of specific lysis were obtained using C3 or 13-2 cells; only the results obtained with the C3 cells are shown.

In a dose-response experiment the difference in CTL activity seen between

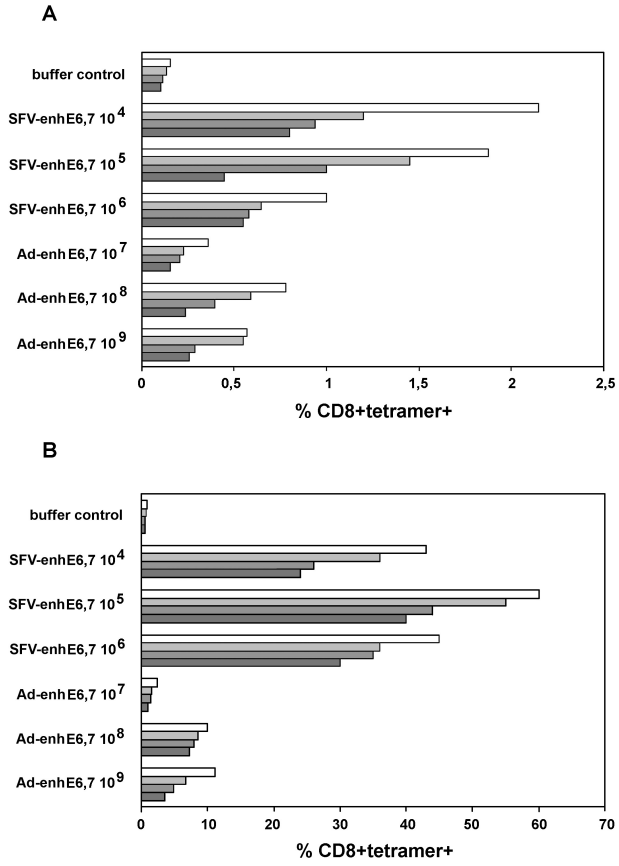

Figure 2. Dose response effect of Ad-enhE6,7 and SFV-enhE6,7 prime-boost immunizations

Mice were immunized and boosted i.m. with 10^4, 10^5 and 10^6 SFV-enhE6,7 or with 10^7, 10^8 and 10^9 Ad-enhE6,7 or with buffer (in each group 4 mice). Spleen cells, isolated one week after the booster immunization were either stained directly (A) or after a 7-day in vitro restimulation (B), with a FITC-labelled monoclonal antibody against $CD8^+$ and a PE-labelled HPV16 $E7_{49-57}$-specific MHC class I tetramer and analyzed by flowcytometry. Data represent percentages of $E7_{49-57}$ tetramer-positive $CD8^+$ cells of individual mice (A and B). In chart C typical flow cytometry data are shown of spleen cells either stained directly upon isolation or after a 7-day in vitro restimulation of buffer control mice or mice immunized with 10^9 Ad-enhE6,7 or 10^5 SFV-enhE6,7. Indicated is the percentage of $tetramer^+$ T cells in the $CD8^+$ T cell population. Figures A and B shown results of a typical experiment, repeated twice. Responses in A and B in all individual groups of SFV or Ad immunized mice were significantly different from the PBS control group ($p=0.03$).

Ad-enhE6,7 and SFV-enhE6,7 was also reflected in the frequencies and the proliferative capacity of precursor CTLs induced (Figure 2). Prime-boost immunizations with as few as 10^4 SFV-enhE6,7 particles induced considerable levels of HPV-specific CTL precursors (Figure 2A) that upon in vitro restimulation readily expanded (Figure 2B). The very low level of pCTLs induced upon immunization with 10^7 Ad-enhE6,7 could not be expanded significantly above background levels by the 7-day restimulation protocol. Immunizations with 10^8 and 10^9 did results in detectable levels of pCTLs that expanded upon restimulation. Yet, while in this experiment pCTLs isolated from SFV-immunized mice expanded to approximately 30-60% of the $CD8^+$, pCTLs isolated from Ad-immunized mice expanded to approximately 10% of the $CD8^+$ T cell population (Figure 2B). To illustrate these responses Figure 2C shows typical flow cytometry data of spleen cells either stained directly upon isolation or after a 7-day in vitro restimulation of mice immunized with 10^9 Ad-enhE6,7, 10^5 SFV-enhE6,7 or buffer.

For SFV immunizations we previously demonstrated that the route of immunization determines to a large extent the outcome of the immune response evoked, i.v. and i.m immunizations being significantly more efficient that i.p. or s.c immunization.[3] To determine the optimal route of injection for adenoviral immunization, $5x10^8$ Ad-enhE6,7 particles were administered via different routes (i.m., i.p. or s.c), twice with a two-week interval. As intravenous injection of rAd is hepatotoxic, Ad-enhE6,7 was not administered via this route.[19;20] Only upon

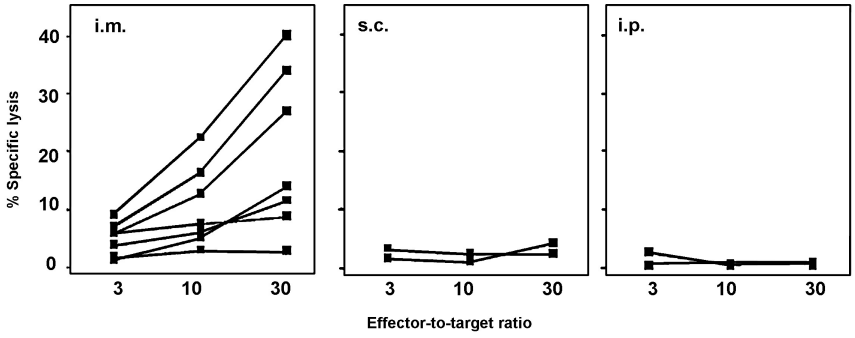

Figure 3. Influence of the route of immunization of Ad-enhE6,7 on the induction of CTL responses

Mice were immunized i.m. (n=7), s.c. (n=2), i.p. (n=2) and boosted via the same route as the primary immunization, with $5x10^8$ Ad-enhE6,7 or PBS as control. After 8 days of *in vitro* restimulation, the resulting effector cells were tested for cytolytic activity against C3 target cells in triplicate well assay. The levels of cytolysis at different effector-to-target ratios are shown. Less than 2% of lysis at an effector-to-target-ratio of 30:1 was observed in mice injected with PBS or SFV-LacZ (not shown). Shown are the results of an experiment repeated twice.

i.m. immunization low but detectable CTL activity was induced; s.c. and i.p. immunization did not induce detectable CTL activity (Figure 3). Although the bulk CTL analysis did not reveal any CTL activity, tetramer analysis demonstrated low but detectable pCTL frequencies by s.c. immunization. Upon i.p. immunization pCTLs frequencies, if induced, were below detection limit (not shown).

Therapeutic efficacy of immunization with Ad-enhE6,7 versus SFV-enhE6,7

We next determined whether the observed difference in CTL activity induced by both vectors is also reflected in the therapeutic efficacy in eradicating established tumours. In the following anti-tumor experiments, Ad-enhE6,7 was compared to i.v. injected SFV-enhE6,7 as our gold standard. We previously demonstrated that i.m. and i.v. SFV-enhE6,7 immunizations resulted in similar levels of pCTL frequencies, CTL activity (also see figure 1) and anti-tumor responses.[3] Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells and subsequently immunized and boosted twice with increasing doses of Ad-enhE6,7 or SFV-enhE6,7 particles. All control mice, either injected i.m. with buffer or with Ad-Luc, developed tumours within 14 days after tumour inoculation (Table 1). As shown in Table 1, 60% of the mice immunized and boosted with $1x10^9$ Ad-enhE6,7 i.m. on days 7, 14 and 21 after tumour inoculation eradicated the tumour and subsequently remained

Table 1. Therapeutic efficacy of immunization with Ad-enhE6,7 versus SFV-enhE6,7

Immunization	Dose	Days after challenge	Number of mice	% Tumor free
Buffer		7-14-21	8	0%
	1x10e4	7-14-21	10	40%
	1x10e5	7-14-21	10	60%*
SFV-enhE6,7	1x10e6	7-14-21	10	100%*
	1x10e6	14-21-28	5	40%
	1x10e7	7-14-21	5	20%
	1x10e8	7-14-21	10	40%
Ad-enhE6,7	1x10e9	7-14-21	10	60%*
	1x10e8	14-21-28	5	0%
Ad-Luc	1x10e9	7-14-21	4	0%

[a] Mice were immunized and boosted twice with SFV-enhE6,7 i.v., Ad-enhE6,7, Ad-Luc or buffer i.m. after tumor inoculation according to the schemes and dosages as indicated.
* Significant different ($p<0.01$) compared to buffer control group using Chi-square analysis.

tumour-free for the next 2 months. Upon immunization with $1x10^8$ and $1x10^7$ Ad-enhE6,7, 40% and 20% of the mice, respectively, cleared the tumour and remained tumour-free. In contrast, all mice immunized and boosted with $1x10^6$ SFV-enhE6,7 i.v. on days 7, 14 and 21 after tumour inoculation eradicated the tumour. When immunizations were initiated as late as 14 days after the tumour-inoculation, all mice treated with $1x10^9$ Ad-enhE6,7 developed a tumour, whereas still 40% of the mice treated with $1x10^6$ SFV-enhE6,7 eradicated the tumour and remained tumour-free.

Thus, the observed difference in CTL induction is also reflected in the therapeutic effect, i.e. SFV-enhE6,7 immunization has a significantly better therapeutic effect compared to Ad-enhE6,7 immunization. Even with a 1000-fold lower dose of SFV-enhE6,7 all mice efficiently eradicated the tumour compared to 60% in the group treated with Ad-enhE6,7.

Effect of $CD4^+$ and/or $CD8^+$ T cell depletion on the anti-tumour effects of Ad-enhE6,7 and SFV-enhE6,7

Although not as effective as SFV-enhE6,7, immunization of mice with Ad-enhE6,7 did generate anti-tumour responses. Considering the low levels of antigen-specific CTLs induced upon Ad-enhE6,7 immunizations, we investigated whether different lymphocyte subsets might be involved in the anti-tumour effect evoked by rAd and rSFV. To determine the actual effector cells involved in the anti-tumour effect seen in mice immunized with Ad-enhE6,7 or SFV-enhE6,7, we *in vivo* depleted T cell subsets using monoclonal antibodies. Mice were immunized and subsequently depleted of $CD4^+$ T cells, $CD8^+$ T cells or both T cell populations. Next, the mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells. During the experiment, the depletion of the T cell subsets was maintained by weekly injections with antibodies. As shown in Figure 4A, all mice in the control group developed tumors within 14 days after tumour inoculation. In the group of immune-competent mice, 7 of 7 mice receiving $5x10^6$ SFV-enhE6,7 were protected from tumour-outgrowth (Figure 4B), whereas in only 2 of 7 mice immunized with $5x10^8$ Ad-enhE6,7 tumour-outgrowth was prevented (Figure 4F). When CD4+ T cells were depleted, all mice immunized with SFV-enhE6,7 and again only 2 of 7 mice immunized with Ad-enhE6,7 were protected (Figure 4B and 4G, respectively). In contrast, in the groups of mice depleted of CD8+ T cells, 7 of 7 mice immunized with SFV-enhE6,7 grew tumours within 20 days after TC-1 challenge (Figure 4D) and all mice immunized with Ad-enhE6,7 developed tumours within 14 days. (Figure 4H) After depletion of both CD4+ and CD8+ T cells in both the rSFV-immunized and the rAd-immunized group, tumours grew comparable to the control group (Figure 4E,I).

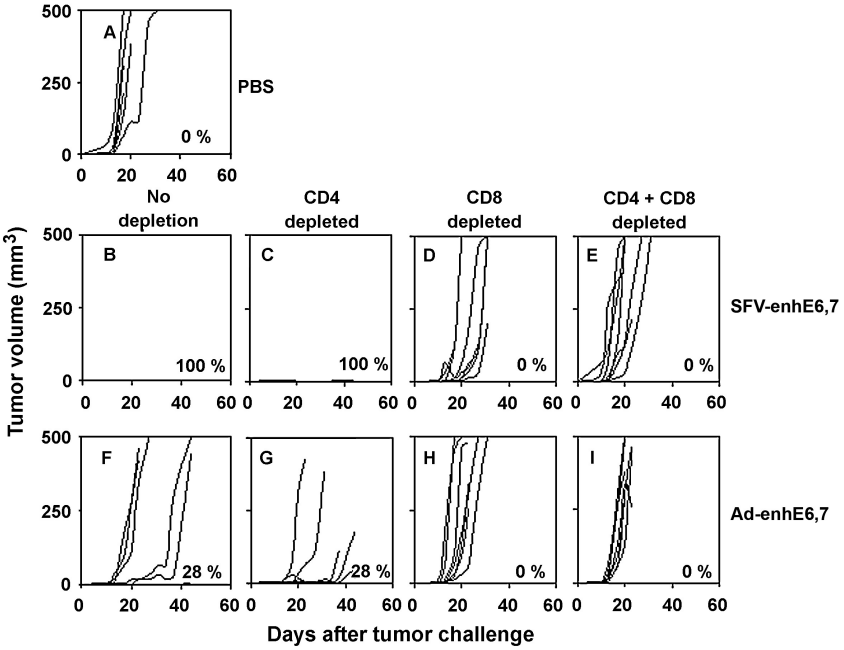

Figure 4. Effect of CD4$^+$ and/or CD8$^+$ T cell depletion on the anti-tumor effects of Ad-enhE6,7 and SFV-enhE6,7

Mice were immunized and boosted with 5x10^6 SFV-enhE6,7 i.v. (panel B, C, D, and E) or 5x10^8 Ad-enhE6,7 i.m. (panel F, G, H, and I). The number of mice per group was 7. Six days after the booster injection and six days before tumor inoculation *in vivo* antibody depletion was initiated. The *in vivo* depletion was performed by i.p. injection of 200 µg/mouse of anti-CD4 (panel C and G), anti-CD8 (panel D and H) or both (panel E and I) for three consecutive days followed by a weekly injection. One week after the initiation of the depletion, the mice were challenged s.c. in the neck with 2x10^4 TC-1 cells. As controls, mice were left without depletion after immunization with PBS i.m. (panel A), SFV-enhE6,7 (panel B) or Ad-enhE6,7 (panel F) before tumor-challenge. The percentages indicate the percentage of tumor-free mice for each treatment at day 40 after tumor inoculation. At a tumor volume of approximately 500mm^3, the mice were sacrificed. The experiments without CD8 and CD4 depletion were repeated at least three times. The depletion experiment was performed once. Results in panel B and C were significantly different from panel A ($p<0.05$), and panel D and E statistically different from panel B ($p<0.05$). The results of the Ad-enhE6,7 immunized groups were not significantly different from the PBS control.

These data indicate that, as expected, CD8+ T cells are the major effector cells involved in the observed anti-tumour effects induced by rSFV as well as rAd immunizations. Furthermore, CD4+ T cells may play a minor role in the anti-tumour response in mice immunized with rSFV as tumour growth was slightly delayed in the group only depleted of CD8+ T cells compared to the PBS control group and the group depleted of both T cell populations.

Luciferase activity induced upon i.m. injection with Ad-Luc and SFV-Luc.
Since immunization with Ad-enhE6,7 results in a significantly lower CTL and anti-tumour activity compared to SFV-enhE6,7 immunizations, we investigated if the amount of transgene product expressed *in vivo* could possibly explain this difference. Therefore, we performed an *in vivo* transfection experiment with Ad-Luc and SFV-Luc. Since the time-point of maximal expression of the transgene product after rSFV and rAd injection differs, we determined the expression level of luciferase at 24 hours after SFV-Luc injection and at 48 hours for Ad-Luc.[21;22] As shown in Figure 5, comparable levels of luciferase activity were measured in the muscles of the mice injected with $1x10^6$ SFV-Luc and $5x10^8$ Ad-Luc.

In the above immunization studies all mice received a booster immunization. It is known that rAd elicits a strong anti-vector response. Anti-vector responses elicited by rSFV are not well documented. We therefore determined the level of recombinant protein produced by a second injection injection with rAd or rSFV. To this end, mice were pre-injected i.m. with rAd or rSFV expressing β-galactosidase (i.e. Ad-LacZ and SFV-LacZ) two weeks before injection of Ad-Luc or SFV-Luc,

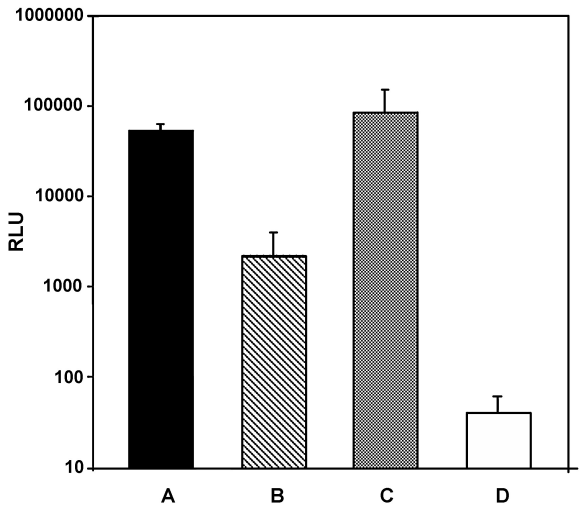

Figure 5. Luciferase activity induced upon i.m. injection with Ad-Luc and SFV-Luc
Luciferase activity in the muscles of mice injected i.m. with $1x10^6$ SFV-Luc (n=4, A) or $5x10^8$ Ad-Luc (n=4, C). To measure anti-vector responses mice were pre-injected with SFV-LacZ and Ad-LacZ two weeks before injecting SFV-Luc (n=2, B) and Ad-Luc (n=2, D), respectively. The muscles were isolated 24h after the i.m. injection of SFV-Luc and 48h after Ad-Luc. Luciferase activity was measured as described in the Materials and Methods section. Data represent the average (±SD) of relative light units (RLU) measured of a typical experiment of two. The background RLU is shown as a horizontal dotted line. Statistical value: bars A vs B: p=0.01; bars C vs D: p=0.01; bars A vs C not significant and bars B vs D: p=0.03.

respectively. Pre-injection of mice with Ad-LacZ resulted in a more than 3-log decrease in luciferase expression compared to naïve Ad-Luc injected mice. In mice injected with SFV-LacZ first and with SFV-Luc next, luciferase activity was decreased by 1 to 2 orders of magnitude compared to SFV-mediated luciferase expression in SFV-naïve mice (Figure 5).

Single versus prime-boost immunizations with Ad-enhE6,7 and SFV-enhE6,7.

To analyse the efficacy of a booster immunizations of Ad-enhE6,7 and SFV-enhE6,7, mice were either injected with $5x10^8$ Ad-enhE6,7 and 'boosted' two weeks later with buffer or the other way around, or mice were primed and boosted with $5x10^8$ Ad-enhE6,7. Other groups of mice were similarly immunized with SFV-enhE6,7. While the booster immunization did not significantly increase pCTL frequencies of single Ad-enhE6,7 immunized mice, the pCTL frequency of single SFV-enhE6,7 immunized mice increased significantly from approximately 0,5% to 1,5% (Figure 6).

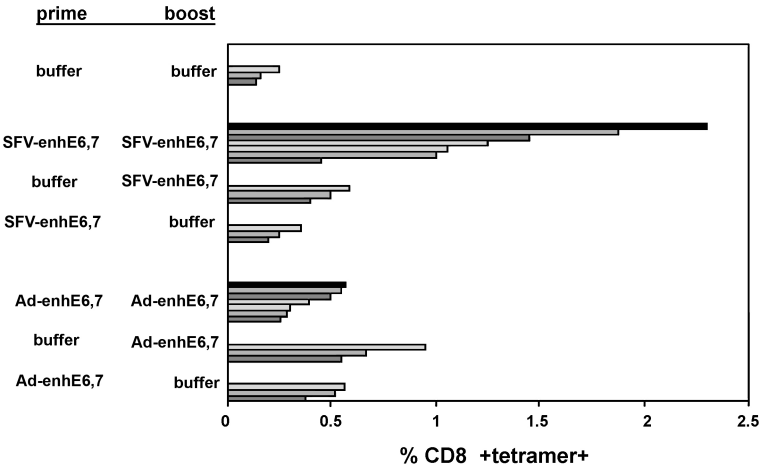

Figure 6. Single versus prime-boost immunizations with Ad-enhE6,7 and SFV-enhE6,7. Mice were either injected with $5x10^8$ Ad-enhE6,7 followed by an injection with buffer two weeks later (n= 3), a second group of mice was first injected with buffer followed by $5x10^8$ Ad-enhE6,7 (n=3), and a third group of mice was injected twice with a two-week interval with $5x10^8$ Ad-enhE6,7 (n=7). Other groups of mice were similarly immunized i.m. with SFV-enhE6,7. One week after the injection, spleen cells were isolated and pCTL frequencies were determined with a FITC-labelled monoclonal antibody against $CD8^+$ and a PE-labelled HPV16 $E7_{49-57}$-specific MHC class I tetramer and analyzed by flowcytometry. Data represent percentages of $E7_{49-57}$ tetramer-positive $CD8^+$ cells of individual mice of a typical experiment repeated twice. Precursor frequencies in groups of mice immunized and boosted with SFV-enhE6,7 or with Ad-enhE6,7 were significantly different from the buffer control group ($p=0.017$). All other groups were not significantly different from the PBS control group.

DISCUSSION

In this study we compared the efficacies of rSFV and rAd as vectors for the induction of cellular and anti-tumor responses against HPV-induced cervical cancer. We previously demonstrated that immunizations with rSFV-enhE6,7 result in very potent CTL and anti-tumor responses in murine animal models.[1-4] However, rSFV has not yet been evaluated in humans hampering a facile introduction of SFV-based immunotherapeutic strategies into the clinic. On the other hand, rAd vectors have already been used in numerous clinical studies against a variety of infectious diseases and tumors.[12-14;16] Thus, as rAd immunizations could potentially be a good alternative for the immunotherapy of cervical cancer. In this study we compared the efficacies of both vector systems.

Here we demonstrate that immunization with SFV expressing the HPV E6 and E7 antigens results in two-fold higher E7-specific CTL precursor frequencies compared to immunizations with 100-fold more infectious particles of an adenoviral vector encoding the same antigens. The therapeutic efficacy of both vectors in eradicating established tumors was studied in a murine HPV-tumor model. Despite the low levels of CTL induced upon immunization with Ad-enhE6,7, up to 60% of the mice eradicated the tumor. In line with our previous studies, all mice immunized with a 1000-fold lower dose (i.e. $1x10^6$) of SFV-enhE6,7 eradicated the tumors.

To explore the possible mechanisms that might explain the difference in efficacy between both vectors we determined i) the role of $CD4^+$ and $CD8^+$ T cells in the anti-tumor responses elicited, ii) the protein expression levels of both vectors, and iii) the influence of possible anti-vector responses on booster injections. The role of $CD4^+$ and $CD8^+$ T lymphocytes in the anti-tumor response was investigated by determining the efficacy of the immunizations in the presence or absence of these T cell subsets. Mice were immunized and subsequently depleted of their $CD4^+$ T cells, $CD8^+$ T cells or both T cell populations using antibodies. Subsequently, the mice were challenged s.c. with tumor cells. Upon depletion of $CD8^+$ T cells in both the rSFV- and the rAd-immunized group, mice were no longer protected against tumor growth. Notably, in the rSFV-immunized group depletion of only the $CD8^+$ T cells resulted in a slight delay of tumor growth of 3-5 days compared to the rSFV-immunized group depleted of both T cell populations and the PBS immunized group, suggesting that also $CD4^+$ T cells may have some anti-tumor effect. However, this delay, which was not observed in the rAd treated group, was not significant, and therefore most likely does not explain the difference in efficacy between both vectors. Depletion of only the $CD4^+$ T cells did not affect the anti-tumor responses elicited with both vector systems. Thus, $CD8^+$ T cells seem to

be the main effector cells involved in the protection against tumor growth elicited both by rSFV and rAd. [23-25]

As the lymphocyte subsets can not explain the difference in efficacy, we next determined the amount of antigen expressed *in vivo* with rSFV and rAd. For this, the expression of luciferase after i.m. injection of Ad-Luc and SFV-Luc was determined. It should be noted that we previously demonstrated that i.m. and i.v. SFV-enhE6,7 immunizations resulted in similar levels of pCTL frequencies, CTL activity and anti-tumor responses.[3] As the time-point of maximal expression of the transgene product after rSFV and rAd injection differs, luciferase expression level was determined at 24 hours after SFV-Luc and at 48 hours after Ad-Luc injection.[21;22] Intramuscular injection of $1x10^6$ SFV-Luc and $5x10^8$ Ad-Luc resulted in comparable levels of luciferase in the muscles of the mice at these given time points. It should however be noted that two to three days after SFV infection, cells die through apoptosis, and hence transgene expression stops. [21] In contrast, transgene expression in cells infected with rAd may lasts for several weeks.[22] Thus the total amount of transgene produced by rAd is much higher than that produced with rSFV. One could therefore hypothesize that, as a consequence of this long-term production of antigen, immunization with rAd should be more efficient than rSFV. Yet, despite the short expression of antigen induced with rSFV compared to rAd, the immune responses elicited with rSFV immunization are more effective in eradicating tumor cells. Although not determined in this study one could also envision that the rate of degradation of the E6,7 fusion protein may differ depending on the expression system.

Pre-existing anti-vector immunity represents a major problem in the development of vector-based vaccines. Anti-adenovirus serotype 5 (Ad5) immunity has already been shown to substantially suppress the immunogenicity of recombinant Ad5 (rAd5) vector-based vaccines. [26-28] Neutralizing antibodies reduce transfection of the adenoviral vectors by cells, and thus expression of the transgene product, which in turn impacts the resulting transgene product-specific immune response. In this study we confirm these studies by demonstrating that that anti-vector responses elicited by prior injections with immune-activating doses of our rAd reduce transgene (luciferase) expression of a subsequent injection with rAd more than 3-log. Anti-vector responses elicited by rSFV are not very well documented. Berglund *et al.* [29] showed that upon immunization with SFV the immune responses against SFV did not disable boost responses by subsequent immunizations with the same vector. Moreover, booster immunizations are indispensable for the induction of long-term memory responses.[1] Despite this, here we demonstrate that prior injections with rSFV at dosages required for immune activation also

reduce transgene expression of subsequently injected rSFV. Yet, in contrast to the almost complete inhibition observed with rAd, preinjection with rSFV results in a 1-2 log decrease. Currently we are further investigating the mechanisms and kinetics of anti-vector responses in rSFV immunizations.

It is known that the mechanism of the activation of an immune response by these vectors differs. Our own studies strongly suggest that the immune response evoked by SFV immunizations does not occur via direct priming of DCs but via cross-presentation of antigen by DCs that have taken up apoptotic rSFV transfected cells.[30] Recently, Chen *et al.*[31] confirmed that infection of DCs with rSFV *in vitro* indeed is very inefficient, and provided further proof that cross-priming is the main mechanism by which immunity to a rSFV is generated. An other important feature of rSFV is that infection of cells with SFV results in the formation of dsRNA intermediates that are known for their immunopotentiating capacity.[32].[33] Mercier *et al.*[34] compared the immune response induced by an i.m. injection of rAd alone with that induced by cells transduced *ex vivo* with the rAd. They demonstrated that in muscle, Ad-transduced myoblasts and endothelial cells are very poor inducers of antigen-specific CD8+ T cell response either by direct stimulation or by cross-priming, and that transduction of DCs is essential for induction of a CD8+ T cell response. Yet, DCs do not express the primary Ad receptor, coxsackie-adenovirus receptor (CAR). As a consequence Ad-mediated transduction of DCs is inefficient.[35] Furthermore, Rea *et al.*[36] showed that DCs activated *in vitro* by rAd lack a high level of CD83 expression and IL-12 production, and therefore, are inhibited to fully mature and polarize toward a Th1-inducing phenotype. This may explain why higher dosis of rAd5 compared to rSFV are needed to elicit effective immune responses. Recently, De Gruijl *et al.* [37] demonstrated that the rAd35 vector, which uses CD46 as a high-affinity receptor which is expressed on human DCs, does infect migrated and mature CD83+ cutaneous DCs with high efficiency when delivered intradermally in an established human skin explant model. They furthermore demonstrated that upon intracutaneous delivery of a rAd35 vaccine, emigrated DC functionally express and process encoded antigenic epitopes and were capable of activating specific CD8+ effector T cells.

Apart from differences in the activation pathway leading to an immune response, the induction of memory T cells generated upon immunization with both vectors might also explain the differences in efficacy of immunization with rAd compared to rSFV. Immunization with viral vector may generate effector T cells against the encoded antigen. Differentiation of these effector T cells into memory T cells follows a linear pathway.[38] As mentioned above, we showed that for SFV-enhE6,7 immunizations, boosting with SFV-enhE6,7 is required for the

induction of CTL memory, resulting in high levels of CTL activity up to 12 months after immunization.[1;2] A single immunization results in a rapid decrease in CTL activity within weeks after the immunization.[1] Following immunization with rAd one can envision two possible mechanisms by which this differentiation pathway into memory T cells could be disturbed. First the strong anti-vector response induced after immunization with rAd prohibits the effect of boosting, and hence a good CTL memory. Secondly, the prolonged expression of antigen with rAd results in effector T cells, which fail to acquire the key properties of memory cells. These effector T cells are maintained for some time and mediate immediate (partial) protection from a challenge. However, no functional central memory T are generated. As a consequence, upon antigen removal, effector T cells disappear and memory T cells do not develop.[39] The balance between too little, just right and too much stimulation determines the induction of memory T cells that acquire memory traits, including the ability to undergo homeostatic proliferation, rapid proliferation upon secondary challenge and the ability to produce cytokine.[40] Our studies suggest that the SFV vector system possesses the ability to generate the "just right" level of stimulation.

In this study we only evaluated immune responses against E7 although the vaccines encodes both E6 and E7. In a separate study we demonstrated that in patients with cervical cancer or premalignant cervical lesions E6 and E7 responses are present.[41] We recently demonstrated that these responses in patients are largely inhibited by the presence of regulatory T cells. After in vitro depletion of regulatory T cells PBMC readily respond (Interferon-gamma release) to both E6 and E7 protein in 60-80% of the patients.[42]

In conclusion, the SFV vector system proved significantly more immunostimulating than the adenoviral type 5 vector system. As, immunization with SFV-enhE6,7 resulted not only in 2-fold higher pCTL frequencies and significantly higher levels of CTL activity, also a significantly superior therapeutic effect was seen after SFV-enhE6,7 immunization requiring 100-1000-fold lower doses compared to Ad-enhE6,7 immunization. Together with the high level of biosafety of the SFV vector system and the absence of pre-existing neutralizing antibodies in humans it seems worthwhile to tackle the severe regulatory hurdles that have to be taken for the evaluation of this 'novel' vector system in humans.

MATERIALS AND METHODS

Cell lines

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (#CCL-10). The cells were grown in GMEM (Invitrogen, Breda, The Netherlands) containing 5% fetal calf serum. C3 cells, 13-2 cells, and TC-1 cells were kindly provided by Dr. C. Melief and Dr. R. Offringa (Leiden University, The Netherlands). The C3 cell line was derived from C57Bl/6 (H-2^b) embryonic cells transfected with a plasmid containing the complete HPV16 genome.[43] The 13-2 cell line was generated from C57Bl/6 (H-2^b) embryonic cells transfected with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF).[44] The TC-1 cell line was generated from C57Bl/6 primary lung epithelial cells with a retroviral vector expressing HPV16 E6E7 plus a retrovirus expressing activated c-Ha-ras.[45] C3, 13-2 and TC-1 cells were grown in IMDM with Glutamax-I (Invitrogen) supplemented with 10% fetal calf serum and penicillin and streptomycin (Invitrogen; 100 U/ml and 100 μg/ml, respectively). TC-1 medium was furthermore supplemented with non-essential amino acids (Invitrogen; 100-fold dilution), sodium pyruvate (Life Technologie; 1 mM) and Geneticin G418 Sulphate (Roche, Germany; 5mg/ml).

Mice

Specific-pathogen-free female C57Bl/6 mice (Harlan CPB, Zeist, The Netherlands) were between 6 and 10 weeks of age at the start of the immunization protocols.

Production, purification, and titer determination of rSFV

The production, purification, and titer determination of recombinant SFV-enhE6,7, SFV-LacZ and SFV-luciferase was performed as described previously.[1] In short, pSFV3-enhE6,7, pSFV3-LacZ, and pSFV3-luciferase were produced using pSFV-Helper 2 and quantified using BHK 21 cells. pSFV3-enhE6,7 encodes an enhanced expression of a fusion product of E6 and E7 of HPV type 16.

Production of rAd5

All recombinant vectors were constructed through homologous recombination in Escherichia Coli using the AdEasy system.[44] The E6,7 fusion protein was isolated from the pHelix eE6,E7 plasmid[1] using restriction enzymes XbaI and KpnI and cloned into the AdTrackCMV plasmid using the same enzymes. This resulted in expression of E6,7 under control of the CMV promoter. The recombinant E1 and E3 deleted vector was grown on 293 cells and purified in HEPES/sucrose buffer,

pH 8.0 according to conventional double CsCl gradient centrifugation method.. The number of viral particles was calculated from the optical density at 260 nm (OD260).[47] Infectious units were determined by limiting dilution assay. In short, 293 human embryonic kidney cells were plated at 10,000 cells/ well in a 96 wells plate. Next day, 100 µl of 10-fold dilutions of the sampled media were added to the wells in 5- or 10-fold. Cytopathic effect was monitored every 2-3 days and scored at day 14. Plaque forming units (pfu) were calculated according to standard procedures. This vector contains green fluorescent protein (GFP) as reporter genes under control of cytamegalovirus (CMV) promoter.[46]
Initial experiments were performed using an adenovirus with two expression cassettes (CMV-Green Fluorescent Protein and CMV-Luciferase; AdGFP-Luc)[48.]

Immunizations
For CTL analysis, mice were immunized with either varying doses of infectious particles of SFV-enhE6,7 i.v. or i.m. or with Ad-enhE6,7 i.m., i.p. or s.c., followed by one booster immunization with a two-week interval. As negative controls, mice were injected with dialysis buffer, PBS or Ad-Luc.

CTL assay
Seven to ten days after immunization, spleen cells were isolated and cocultured with irradiated (100 Gy) TC-1 cells in a ratio of 25:1, in 25 cm^2 culture flasks, placed upright. After a one-week in vitro restimulation, cells were harvested and a CTL assay was performed by a standard 4-hr ^{51}Cr release assay in triplicate. Target cells (13-2 cells and C3 cells) were labeled for 1 h with 3.7 MBq $^{51}Cr/10^6$ cells in 100 µl medium (MP Biomedicals, Inc., Irvine, USA). The mean percentage of specific ^{51}Cr-release was calculated according to the formula: % specific release = [(experimental release-spontaneous release)/(maximal release-spontaneous release)] cpm x 100. The spontaneous ^{51}Cr-release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the mean. Similar levels of specific lysis were obtained using C3 or 13-2 cells; only the results obtained with the C3 cells are shown.

MHC class I tetramer staining and FACS analysis
To analyze the number of $CD8^+$ T cells specific for the HPV 16 $E7_{49-57}$ peptide RAHYNIVTF we used K^b-RAHYNIVTF tetramers produced in the laboratory of Dr. Ton Schumacher (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Spleen cells were washed with FACS buffer (PBS containing 0,5% BSA and 0,02% sodium azide) and stained with FITC-conjugated anti-CD8a (Pharmingen) together

with PE-conjugated K^b-RAHYNIVTF tetramers for 20 minutes at 4°C. Spleen cells were washed three times and analyzed by flow cytometry (FACSCalibur, Beckton Dickinson). Live cells were selected based on propidium iodide exclusion.

Luciferase assay

To determine luciferase activity, organs were collected, immediately frozen in liquid nitrogen and kept at -80°C. Organs were crunched into powder in a mortar on dry ice. For luciferase determination the Luciferase Reporter 1000 Assay System (Promega, Cat.#E4550),was used.Firefly luciferase, a monomeric 61kDa protein, catalyzes luciferin oxidation using ATP-Mg^{2+} as a cosubstrate. In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined. The Luciferase Assay System incorporates coenzyme A(CoA) for improved kinetics, allowing greater enzymatic turnover resulting in increased light intensity that is nearly constant for at least 1 minute. In brief, the material was lysed in lysis buffer (Promega) (300 µl / 0,1 gram of tissue). The suspension was frozen and thawed three times with vigorous vortexing in between. To remove cell debris the suspensions were centrifused twice in an Eppendorf centrifuge. Immediately before measurement, 4 µl of the supernatant of the samples was mixed with 36 µl luciferase substrate solution. Luciferase signal was determined in a Lumicount (Packard).

Tumor treatment experiments

Mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells suspended in 0.2 ml Hanks Buffered Salt Solution (Invitrogen). Subsequently, mice were immunized and boosted twice with a one-week interval, with SFV-enhE6,7, Ad-enhE6,7, Ad-Luc or dialyse buffer starting at days 7 or 14 after tumor inoculation. Immunization routes were i.v. for SFV-enhE6,7 and i.m. for Ad-enhE6,7, Ad-Luc and dialyse buffer. The same skilled technician always did tumor measurements. At a tumor volume of approximately 1000 mm^3, the mice were sacrificed.

In vivo depletion of T cell subsets and tumor challenge

For the *in vivo* depletion of $CD4^+$ and $CD8^+$ T cells, the monoclonal antibodies GK1.5 (anti-CD4) and 2.43 (anti-CD8) were used. The hybridoma's were kindly provided by Prof A. Kruisbeek. (Free University Amsterdam, The Netherlands). The monoclonal antibodies (mAbs) were produced by culturing the hybridoma cells in the two-chamber cell culture device CELLine 350 (Integra Biosciences) according to the manufacturer's instruction. For the purification of the mAbs HiTrap™Protein G HP 1ml columns (Amersham Biosciences) were used in an FPLC

system. mAbs concentrations were determined by spectrophotometry at 280 nm using an extinction coefficient of 1,35 mg/ml.

The *in vivo* depletion was performed by i.p. injection of 200 µg/mouse of anti-CD4, anti-CD8 or both for three consecutive days followed by a weekly injection. Depletion of lymphocyte subsets was assessed by flow cytometric analysis of spleen cells using anti-CD4-PE (IQ Products, Groningen, The Netherlands/ Pharmingen) and anti-CD8-FITC (Pharmingen) antibodies. This antibody treatment resulted in 97% depletion of CD8 T cells and 91% depletion of CD4 T cells in spleen. In blood 95% and 83% depletion of CD8 and CD4 T cells, respectively was obtained with this treatment (not shown).This level of depletion was maintained by the weekly injections.

For the tumor challenge experiment, mice were immunized and boosted with $5x10^6$ SFV-enhE6,7 i.v. or $5x10^8$ Ad-enhE6,7 i.m.. Six days after the booster injection and six days before tumor inoculation *in vivo* antibody depletion was initiated. One week after the initiation of the depletion, the mice were challenged s.c. with $2x10^4$ TC-1 cells suspended in 0,2 ml Hank's Buffered Salt Solution (Invitrogen).

Statistical analysis

Data depicted in Figure 4 were statistically analysed using Chi-square testing. The other data with the Mann-Whitney *U*-test.

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CHAPTER 8

Heterologous prime-boost immunization protocols with a recombinant Semliki Forest virus vector and virosomes

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Manuscript submitted for publication

ABSTRACT

In heterologous prime-boost immunization strategies, antigen-specific immune responses are primed by one vector or antigen delivery system and selectively boosted by another system. Such strategies are found to establish higher frequencies of antigen-specific T lymphocytes than homologous prime-boost protocols or single immunizations. Previously, we developed virosomes and recombinant Semliki Forest Virus (rSFV) as systems each capable of inducing strong CTL responses in homologous prime-boost protocols.

Here, we demonstrate that a heterologous prime-boost protocol with rSFV and virosomes results in higher numbers of antigen-specific pCTL in mice than homologous protocols. A virosome prime followed by an rSFV boost was more effective in this respect than vice versa. Evasion of vector-specific immunity appeared to play a role in establishing these high frequencies, as co-induction of vector-specific responses during the prime immunization reduced the frequency of target antigen-specific pCTL after a heterologous booster. However, the induction of high numbers of pCTL did not correlate with improved functional immune responses. Heterologous prime-boosting did not result in CTL with an enhanced responsiveness to *in vitro* antigenic stimulation, nor did it result in improved cytolytic activity or superior anti-tumor responses *in vivo* compared to a homologous protocol with rSFV, although the responses were improved compared to homologous prime-boosting with virosomes.

This study indicates that heterologous prime-boost immunization protocols with rSFV and virosomes do not induce superior CTL responses and underlines the potency of homologous prime-boost protocols involving rSFV alone.

INTRODUCTION

In heterologous prime-boost immunization strategies, an antigen-specific immune response is primed by delivery of the target antigen by one vector or delivery system and selectively boosted by a subsequent immunization using a second, distinct, system[1]. Heterologous prime-boost protocols have been found to establish higher frequencies of antigen-specific $CD8^+$ and $CD4^+$ T lymphocytes than homologous prime-boost immunization protocols or single-immunization regimens[1-4]. Additionally, heterologous prime-boost protocols have been described to generate $CD8^+$ cytotoxic T lymphocytes (CTL) of higher avidity[5] and effector memory $CD8^+$ T lymphocytes, a particularly desirable quality for protective immunity against certain pathogens[6].

Heterologous prime-boost protocols are generally thought to be more effective than homologous protocols because prime-induced immune responses against the vector or delivery system that might limit the booster immunization in homologous prime-boost strategies are circumvented[1;7;8]. Antibodies, induced by the priming immunization, may neutralize the vector or antigen delivery system. Additionally, cellular responses could kill cells that express antigens of the vector or delivery

system[9]. Yet another mechanism related to immunity against the vector or antigen delivery system is immunodominance. During the priming immunization, T lymphocyte responses against epitopes of both the target antigen and the vector or delivery system will be induced. In homologous prime-boost protocols, both of these responses will be stimulated by the booster immunization. A heterologous booster only shares the target antigen with the priming immunization and will therefore preferentially boost the T lymphocyte response against the target antigen. Heterologous prime-boost protocols thereby focus the immune response on immunodominant epitopes of the target antigen[1;4;7;10]. In several recent studies, the efficacy of heterologous prime-boost immunization strategies against, for example, malaria[11;12], HIV[13-15], and tumor antigens[16-18] has been investigated. These heterologous prime-boost strategies, while often inducing increased numbers of antigen-specific IFN-γ producing T lymphocytes, did not always result in improved responses towards the pathogen or tumor cells. In some cases, the effectiveness of the responses *in vivo* was not studied.

We have developed immunization strategies for the treatment of (pre)malignant cervical disease based on a virosomal antigen delivery system[19;20] or based on the recombinant Semliki Forest virus (rSFV) vector system[21;22]. The etiology of cervical cancer involves a persistent infection with a high-risk type of human papillomavirus (HPV)[23]. This virus constitutively expresses the tumor-specific antigens E6 and E7, which are involved in cell transformation, immortalization, and tumorigenicity. The constitutive expression of these unique tumor-specific antigens makes cervical cancer an attractive candidate for immunotherapy. E7-virosomes and especially SFV-enhE6,7 have been shown to be very effective in inducing CTL responses against HPV16 E6- and E7-expressing cells[24-26]. Virosomes, in our studies derived from influenza virus, are reconstituted virus envelopes that retain the cell entry properties of the native Influenza virus[27]. These virosomes can be taken up by professional antigen presenting cells (APC) via receptor-mediated endocytosis. Protein antigens, encapsulated in the virosomal lumen may thus be introduced in the major histocompatibility complex (MHC) class I route of antigen presentation[28]. rSFV is a replication-defective alphavirus vector that consists of a single-stranded RNA molecule encapsidated in recombinant virus particles. Infection of target cells leads to RNA replication and synthesis of a heterologous protein encoded by the recombinant viral genome[29;30]. As rSFV is incapable of infecting dendritic cells (DC)[31], MHC class I presentation of the transgene for the induction of CTL responses proceeds predominantly via antigen transfer from initially transfected cells to professional APC[31;32].

Both virosomes and rSFV are capable of inducing strong CTL responses in

homologous prime-boost protocols. Nevertheless, for immunotherapeutic applications induction of the strongest possible response is desirable. In the current study, we have therefore investigated whether heterologous prime-boosting with virosomes and rSFV represents an even more potent immunization strategy for the induction of CTL responses and anti-tumor activity than homologous protocols.

MATERIALS AND METHODS

Cells

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection (No. CCL-10). BHK-21 cells were grown in GMEM (Invitrogen, Paisley, UK) containing 5% fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen), and 100 g/ml streptomycin (Invitrogen). C3 cells, 13-2 cells and TC-1 cells were a kind gift from Dr C Melief and Dr R Offringa (Leiden University Medical Center, The Netherlands). The C3 cell line is a C57BL/6 (H-2b) embryonic cell transfected with a plasmid encoding the complete HPV16 genome[33]. The 13-2 cell line was generated by transfection of C57Bl/6 (H-2b) embryonic cells with the E1-region of adenovirus type 5 in which the adenoviral E1A epitope SGPSNTPPEI is replaced by a HPV16 E7 CTL epitope, AA 49-57 (RAHYNIVTF)[34]. C3, and 13-2 cells were grown in IMDM (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin. The TC-1 cell line was generated from C57Bl/6 (H-2b) primary lung epithelial cells with two retroviral vectors, one expressing HPV16 E6E7, the other expressing activated c-Ha-ras[35]. TC-1 was cultured in IMDM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 g/ml streptomycin, 10 mM Sodium Pyrumvaat MEM (Invitrogen), Non-essential amino acids (100-fold dilution of an Invitrogen stock), and 30 µM β-Mercapto ethanol.

Mice

Specified pathogen-free female C57BL/6 mice were used at 8 to 10 weeks of age. They were purchased from Harlan CPB (Zeist, The Netherlands) and kept accourding to institute guidelines. All animal experiments were approved by the local Animal Experimentation Ethical Committee.

Recombinant SFV

Recombinant SFV was produced as previously described[25;36]. In brief, the plasmids pSFV3 and pSFV3 containing the β-Gal sequence (pSFV-β-gal) were purchased from Life Technologies[29]. The plasmid pSFV-Helper 2 was kindly provided by

Dr. Peter Liljeström, Stockholm, Sweden[37]. The HPV16 E6 and E7 genes were obtained from the plasmid pRSVHPV16E6E7,which was kindly provided by Dr J Ter Schegget, Amsterdam, The Netherlands[38]. The pSFV3-enhE6,7 plasmid encodes for an enhanced expression of a fusion product of E6 and E7. It was generated by inserting one base pair between the E6 and E7 genes and changing the stop codon TAA of E6 in GAA while, furthermore, a sequence encoding a translational enhancer was cloned in front of the E6,7 fusion construct. The rSFV and the pSFV-Helper 2 plasmids were isolated using the Qiagen midi plasmid purification kit (Qiagen, Inc., Md., USA) and linearized by digestion with SpeI (Invitrogen). RNA was synthesized from the linearized DNA by *in vitro* transcription using SP6 RNA polymerase (GE Healthcare, Piscataway, NJ, USA). rSFV RNA (15 µg) admixed with SFV-Helper-2 RNA (7.5 µg) was electroporated into BHK cells (8×10^6) in 0.8 ml GMEM using the Biorad Gene Pulser® II (two pulses of 850 V/25 µF; Biorad, Hercules, CA., USA). After pulsing, the cells were suspended in 10 ml GMEM and cultured at 37ºC and 5% CO_2 for 36 h. The medium containing rSFV particles was separated from cells and cellular debris by centrifuging it twice in a JA 20 rotor (Beckman, St Paul, MN., USA) at 1800 rpm (that is, 40 000 ×*g* at rmax). The rSFV particles were purified on a discontinuous sucrose density gradient (15%/50% sucrose solution (w/v) in TNE-buffer (50 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 7.4). rSFV was collected from the interface and sucrose was removed by overnight dialysis against TNE-buffer. Finally, the rSFV suspension was concentrated approximately 10-fold (Centricon 30 filter; Millipore, Bedford, MA, USA), quickly frozen in liquid N_2 and stored in aliquots at –80ºC. rSFV particles were activated by incubation with 1/20 volume of α-chymotrypsin (10 mg/ml; Sigma, St. Louis, MO, USA) for 30 min at room temperature to cleave the mutated viral E2 spike protein. α-chymotrypsin was inactivated by the addition of 0.5 volume of aprotinin (2 mg/ml; Sigma). Mice were immunized with $1x10^6$ particles of rSFV in 50 µl i.m.

HPV16 E7 protein production

Recombinant HPV16 E7 protein was produced as described before[39]. The E7 cDNA was amplified by PCR from the vector pX-HPV-16 E7[40] and inserted into the vector pET3a[41], generating the bacterial expression vector. *Escherichia coli* BL21(DE3)pLysS (Stratagene, La Jolla, CA, USA), transformed with pET3a-HPV-16 E7 were grown to an OD_{600} of 0.5, induced for 3 h at 37 °C by adding 0.4 mM IPTG (Biomol, Hamburg, Germany), harvested and resuspended in lysis buffer (50 mM KCl, 20 mM H_2KPO_4, 50 mM DTT, 5% glycerol, 1 complete-mini-EDTA-free protease inhibitor cocktail tablet (Roche, Vienna, Austria)/50 ml lysis buffer, pH 7.8). Cells were lyzed by sonication, centrifuged at 70 000 × *g* for 30 min and the

E7 protein was ammonium sulphate precipitated by adding 60% (v/v) saturated $(NH_4)_2SO_4$ solution to the supernatant. The resulting protein pellet was dissolved in MonoQ low salt loading buffer (150 mM Tris, 10 mM NaCl, 10 mM DTT, 5% glycerol, pH 7.8), dialyzed against MonoQ low salt loading buffer and loaded onto a MonoQ HR10/10 column (GE Healthcare). The bound proteins were eluted from the anion-exchange column with a linear salt gradient (10–1000 mM NaCl) and E7 came off the column at 470 mM NaCl. E7 containing fractions were pooled and loaded onto a pre-equilibrated HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare). The flowrate of the gel filtration buffer (150 mM Tris, 150 mM NaCl and 10 mM DTT, pH 7.8) was set to 1 ml/min. Identity of the E7 protein was confirmed by Western blot. The gel filtration buffer was removed by dialysis against HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) prior to use of E7 protein for the preparation of E7-virosomes.

E7-virosomes

E7-virosomes were prepared as described previously[26]. In short, A/Panama/2007/99 Influenza virus (1.5 µmol of viral membrane phospholipid) was solubilized in 350 µl HNE buffer (5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA) containing 200 mM octa(ethyleneglycol)-*n*-dodecyl monoether ($C_{12}E_8$) (Calbiochem, San Diego, CA, USA) overnight at 0°C. Next, Influenza virus RNA was removed from the preparation by ultracentrifugation of the nucleocapsid. HPV16 E7 protein in 350 µl HNE buffer was added to the Influenza virus supernatant in $C_{12}E_8$ in a final concentration of 0.5 mg/ml. Subsequently, the detergent $C_{12}E_8$ was extracted from the supernatant by incubation with BioBeads SM2 (Bio-Rad, Hercules, CA, USA), leading to the formation of E7-containing virosomes. The virosomes were applied to a discontinuous sucrose density gradient (10%/50%) to separate them from non-encapsulated E7. Sucrose was removed by dialysis against HNE buffer and E7-virosomes were subsequently concentrated by centrifugation in an Amicon Ultra-4 filter device (Millipore, Bedford, MA, USA; 30000 MWCO). Virosomal phospholipid content was determined by phosphate analysis[42] and virosomal protein was determined according to Lowry[43]. For immunization, 50 nmol of E7-virosomes in 50 µl HNE was injected i.m.

MHC class I tetramer staining and FACS analysis

To analyse the number of $CD8^+$ T cells specific for the HPV 16 $E7_{49-57}$ peptide RAHYNIVTF, 10^6 spleen cells were stained with FITC-conjugated anti-CD8a (BD Pharmingen, San Diego, CA, USA) and PE-conjugated Kb-RAHYNIVTF tetramers, (Sanquin, Amsterdam, The Netherlands) for 20 min at 4°C. Spleen cells were

washed three times with FACS buffer (PBS containing 0.5% BSA (Merck, Darmstadt, Germany)) and analyzed by flow cytometry (FACSCalibur from BD Biosciences, Erembodegem, Belgium). Living cells were selected based on propidium iodide exclusion.

CTL assay

Ten days after receiving their last immunization, mice were sacrificed and spleen cell were isolated. The spleen cells were restimulated with irradiated (100 Gy) TC-1 cells at an effector-to-stimulator ratio of 25:1 in 25 cm^2 culture flasks, placed upright. A standard 4 h ^{51}Cr release assay in triplicate determinations was performed after five or seven days of culture. Two days before performing the ^{51}Cr release assay, 4 U/ml of recombinant human IL-2 (Strathmann Biotech, Hamburg, Germany) was added to the target cells. C3 and 13-2 target cells were labeled for 1 h with 3.7 MBq $^{51}Cr/10^6$ cells in 50 µl medium (^{51}Cr was from MP Biomedicals, Asse-Relegem, Belgium). The following formula was used to calculate specific lysis: % specific lysis = (experimental release − spontaneous release) / (maximal release − spontaneous release) × 100. Spontaneous release was determined from target cells incubated without effector cells and maximal release was determined from target cells incubated with medium containing 0.5% Triton X-100. The spontaneous ^{51}Cr release was always <15%. The standard errors of the means of the triplicate determinations were <10% of the value of the mean.

Tumor treatment experiments

For tumor inoculation, TC-1 cells were harvested, washed 3 times with PBS, and suspended in Hanks Buffered Salt Solution (Invitrogen). Mice were inoculated sc in the neck with $2x10^4$ TC-1 cells in 0.2 ml. Subsequently, mice were immunized i.m. 14 days, 21 days, and 28 days after tumor inoculation. Tumor growth was measured twice weekly by palpation. The size of a tumor was calculated using the following formulas: 0.5236 x $diameter^3$ (for a spherical tumor), 0.7854 x $diameter^2$ x length (for a cylindrical tumor).

RESULTS

The effect of a heterologous prime-boost protocol with rSFV and virosomes on CTL activation

To compare the efficacies of heterologous versus homologous prime-boost immunizations with rSFV and virosomes, we first determined the frequencies of the epitope-specific precursor CTL as induced by these protocols. Mice were primed

and boosted 14 days later with rSFV expressing a fusion protein of HPV 16 E6 and E7 (SFV-enhE6,7) or E7-containing virosomes (E7-virosomes) in homologous and heterologous combinations. Based on previous studies[19;25;26], doses that induce strong CTL responses in homologous prime-boost protocols were used; 10^6 SFV-enhE6,7 particles and 50 nmols of virosomal phospholipids (i.e. ~2.5 µg of E7 protein) respectively. Ten days after the booster immunization, the mice were sacrificed and spleens were collected. As determined by tetramer staining using MHC class I tetramers carrying the $E7_{49-57}$ (RAHYNIVTF) peptide, a heterologous prime-boost protocol with SFV-enhE6,7 followed by a booster immunization with E7-virosomes resulted in approximately 2.7% E7-specific $CD8^+$ T lymphocytes (Figure 1A). A prime immunization with E7-virosomes and a subsequent booster immunization with SFV-enhE6,7 resulted in higher frequencies ranging between 4.7% and 6.9%. Both homologous immunization protocols induced precursor frequencies of about 0.7% and a single immunization with SFV-enhE6,7 resulted in

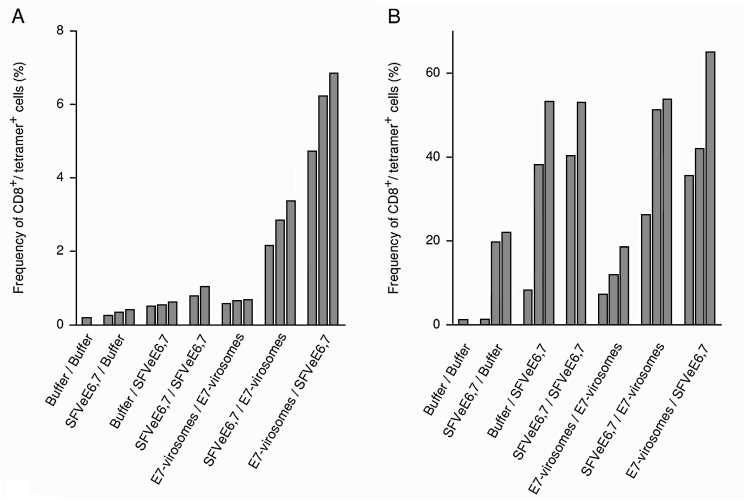

Figure 1: The effect of a heterologous prime-boost immunization protocol on E7-specific pCTL frequencies.

Mice were immunized i.m. with 10^6 SFV-enhE6,7 and injected with buffer i.m. fourteen days later, injected with buffer i.m. and immunized i.m. with 10^6 SFV-enhE6,7 fourteen days later, immunized twice with 10^6 SFV-enhE6,7, treated according to a homologous protocol with 50 nmol E7-virosomes, prime immunized i.m. with 10^6 SFV-enhE6,7 and booster immunized i.m. with 50 nmol E7-virosomes, or prime immunized i.m. with 50 nmol E7-virosomes and booster immunized i.m. with 10^6 SFV-enhE6,7. A control mouse that received two buffer injections was also included. Ten days after the last injection, mice were sacrificed and spleens were isolated. **A**. Freshly isolated splenocytes and **B**. splenocytes after an additional 7-day *in vitro* restimulation were analyzed by flow cytometry after staining with Pe-labeled HPV16 $E7_{49-57}$ carrying MHC class I tetramers and FITC-labeled monoclonal antibodies against CD8. The percentages of tetramer-positive CD8-positive cells of individual mice are shown.

an average frequency of 0.4%. Clearly, a heterologous prime-boost immunization protocol, especially a virosome prime followed by an rSFV boost, resulted in much higher frequencies of E7-specific $CD8^+$ T lymphocytes than either a homologous prime-boost protocol or a single immunization with rSFV.

The potency of an immune response is not merely based on the number of specific cells that is induced. The functional capacity of the induced cells is also of critical importance. To investigate if the precursor CTL can expand, the numbers of E7-specific T lymphocytes were determined after a 7-day antigen-specific *in vitro* restimulation. The number of E7-specific $CD8^+$ T lymphocytes was substantially increased, reaching approximately 47%, in the splenocytes of mice immunized twice with SFV-enhE6,7 (Figure 1B). Both heterologous prime-boost protocols resulted in approximately the same level of E7-specific $CD8^+$ T lymphocytes, although it should be noted that the initial frequency of antigen-specific cells was higher at the start of the *in vitro* restimulation. The level of 0.4% pCTL induced by a single immunization with SFV-enhE6,7 on day 0 followed by a buffer injection on day 14 expanded to about 14%, whereas the 0.4% pCTL induced by a buffer injection on day 0 followed by an immunization with SFV-enhE6,7 on day 14 expanded *in vitro* to 33% on average. Splenocytes from mice immunized twice with E7-virosomes did not reach such high levels. Consistent with a previous study[26], between 7.3% and 18.5% of the $CD8^+$ T lymphocytes were specific for E7 after two immunizations with E7-virosomes and 7 days of *in vitro* restimulation. This experiment shows that E7-specific $CD8^+$ T lymphocytes, induced by a homologous protocol with E7-virosomes have the lowest proliferative capacity. Both heterologous prime-boost protocols induced E7-specific $CD8^+$ T lymphocytes with a high proliferative capacity. E7-specific $CD8^+$ T lymphocytes, induced by a homologous protocol with rSFV, expanded to the same high extent.

The effect of adding irrelevant rSFV to E7-virosomes during a heterologous prime-boost protocol

The absence of an immune response against the vector during the booster immunization is thought to be the main factor contributing to the potency of heterologous prime-boost protocols. Here, we investigated whether the potency of our most potent heterologous prime-boost immunization regime (E7-virosomes followed by SFV-enhE6,7) is due to evasion of prime-induced vector-specific immunity. For that purpose, rSFV expressing an irrelevant antigen (SFV-LacZ) was added to E7-virosomes and this mixture was administered as a priming immunization inducing both E7-specific immunity and SFV-vector-specific immunity. The addition of SFV-LacZ to the E7-virosome prime immunization reduced the

pCTL frequency from 7.8% to 3.9% (Figure 2A). Furthermore, an injection of SFV-LacZ followed by an immunization with SFV-enhE6,7 resulted in about 0.4% E7-specific $CD8^+$ T lymphocytes, whereas a single immunization with SFV-enhE6,7 without pre-injection of SFV-LacZ resulted in up to 0.8%. A homologous prime-boost immunization protocol with SFV-enhE6,7 induced approximately 1.6% E7-specific $CD8^+$ T lymphocytes. These results show that when an irrelevant rSFV vector was added to the E7-virosomes during the priming immunization, the initial induction of pCTL was reduced compared to "clean" heterologous prime-boosting but still considerably higher than the pCTL induction by a homologous prime-boost protocol or single immunization with SFV-enhE6,7. Apparently, the effect on the induction of antigen-specific pCTL is limited and, thus, the potency of heterologous prime-boosting not solely attributable to evasion of vector-specific immunity.

The effect of evasion of vector-specific immunity on the proliferative capacity of the E7-specific $CD8^+$ T lymphocytes was determined by tetramer staining after

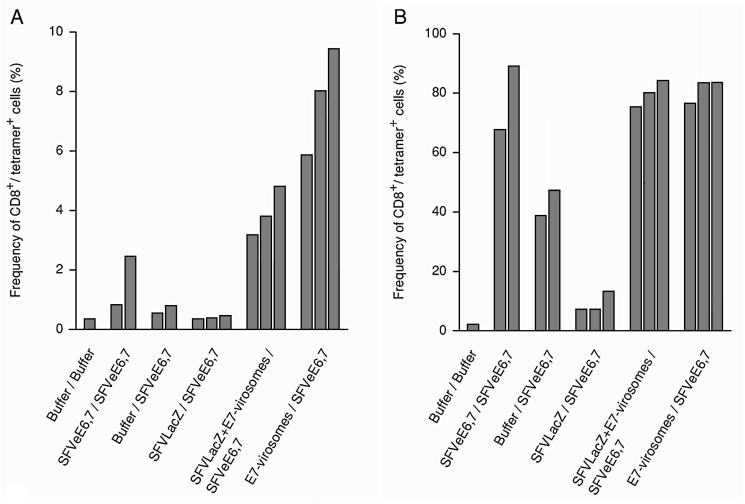

Figure 2: The effect of admixing irrelevant rSFV with E7-virosomes during the prime immunization on the E7-specific pCTL frequencies after heterologous boosting.
Mice were immunized according to a homologous protocol with 10^6 SFV-enhE6,7, injected with buffer i.m. and immunized i.m. with 10^6 SFV-enhE6,7 fourteen days later, pre-injected i.m. with 10^6 SFV-LacZ and immunized with 10^6 SFV-enhE6,7, prime immunized i.m. with 50 nmol E7-virosomes mixed with 10^6 SFV-LacZ and booster immunized i.m. with 10^6 SFV-enhE6,7, or prime immunized i.m. with 50 nmol E7-virosomes and booster immunized i.m. with 10^6 SFV-enhE6,7 fourteen days later. A control mouse that received two buffer injections was also included. Ten days after the last injection, mice were sacrificed and spleens were isolated. **A**. Freshly isolated splenocytes and **B**. splenocytes after an additional 7-day *in vitro* restimulation were analyzed by flow cytometry after staining with Pe-labeled HPV16 $E7_{49-57}$ carrying MHC class I tetramers and FITC-labeled monoclonal antibodies against CD8. The percentages of tetramer-positive CD8-positive cells of individual mice are shown.

7 days of *in vitro* restimulation. After a single immunization with SFV-enhE6,7, E7-specific $CD8^+$ T lymphocytes made up about 43% of the *in vitro* restimulated splenocytes (Figure 2B). A single immunization with SFV-enhE6,7 preceded by an injection with SFV-LacZ, on the other hand, resulted in E7-specific $CD8^+$ T lymphocytes numbers ranging between 7.3% and 13.3%. All prime-boost immunization protocols (homologous and heterologous), including an E7-virosomes + SFV-LacZ prime followed by an SFV-enhE6,7 boost resulted in E7-specific $CD8^+$ T lymphocytes that expanded to approximately 80%. Thus, compared to a standard heterologous prime-boost protocol, the capacity to expand of the pCTL upon *in vitro* restimulation is not reduced when SFV-LacZ is admixed with E7-virosomes during the prime immunization.

The effect of a heterologous prime-boost protocol with rSFV and virosomes on the cytolytic activity of the induced CTL

To determine the cytolytic activity of the antigen-specific CTL induced by a heterologous prime-boost immunization protocol with rSFV and virosomes, a ^{51}Cr release assay was performed. After 7 days of *in vitro* restimulation, the cytolytic activity of splenocytes from all immunization protocols was of the same magnitude, ranging between 72% and 84% at an effector cell to target cell (E:T) ratio of 30 to 1 (Figure 3A). Only the cytolytic activity of splenocytes induced by a homologous prime-boost protocol with E7-virosomes appeared to be slightly lower. As cytolysis was determined after 7 days of restimulation, these results are conceivably due to the fact that similar maximum levels of CTL are present after long-term *in vitro* restimulation.

In an attempt to detect differences between homologous and heterologous regimens, cytolysis was also determined after 5 days of *in vitro* restimulation. Unlike after 7 days, after 5 days of restimulation, the antigen-specific T lymphocytes have not expanded optimally, allowing determination of differences in their intrinsic cytolytic activity. A homologous prime-boost protocol with E7-virosomes did not induce CTL responses detectable after only 5 days of *in vitro* restimulation (Figure 3B). A homologous protocol with rSFV as well as the heterologous prime-boost protocols and a heterologous immunization protocol with irrelevant rSFV incorporated in the prime resulted in the same levels of cytolytic activity after 5 days of *in vitro* restimulation. Thus, although heterologous prime-boost immunizations with rSFV and virosomes result in higher numbers of specific CTL, apparently such protocols do not result in immune responses that are more potent in killing tumor cells *in vitro*. Additionally, rSFV is found to be more potent than virosomes as the immunization protocols that incorporate rSFV induce stronger CTL responses

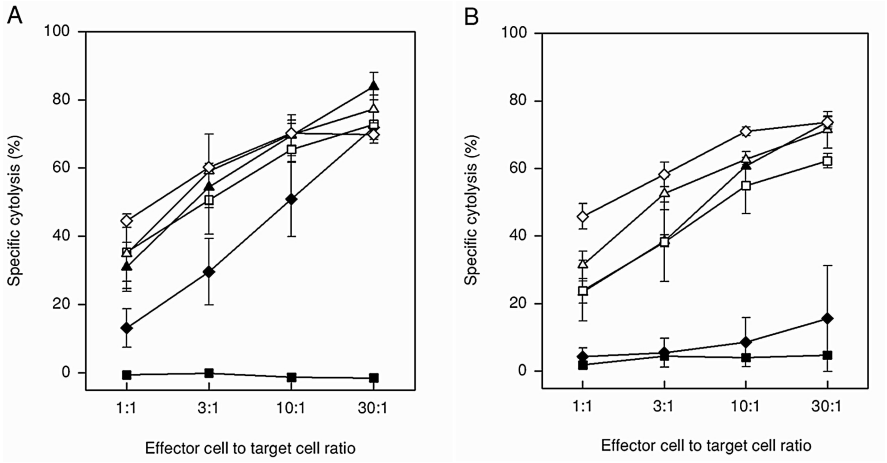

Figure 3: The effect of a heterologous prime-boost immunization protocol on the cytolytic activity.
Mice were primed i.m. with 10^6 SFV-enhE6,7 and boosted i.m. with 50 nmol E7-virosomes (open squares, n=3), primed i.m. with 50 nmol E7-virosomes and boosted i.m. with 10^6 SFV-enhE6,7 (open triangles, n=3), primed i.m. with 50 nmol E7-virosomes mixed with 10^6 SFV-LacZ and boosted i.m. with 10^6 SFV-enhE6,7 (open diamonds, n=3), immunized according to a homologous protocol with 10^6 SFV-enhE6,7 (black triangles, n=2), or treated according to a homologous protocol with 50 nmol E7-virosomes (black diamonds, n=2). A control mouse that received two buffer injections was also included (black squares, n=1). Ten days after the last injection, mice were sacrificed and spleen cells were isolated. After **A**. 7 days or **B**. 5 days *in vitro* restimulation, cytolytic activity against C3 and 13-2 target cells was determined in triplicate well assay. The levels of specific cytolysis at different effector cell to target cell ratios are shown with error bars representing standard deviation.

than a homologous protocol with virosomes. Finally, adding SFV-LacZ with E7-virosomes in the prime immunization of a heterologous prime-boost protocol does not affect specific cytolysis, indicating that evasion of vector-specific immunity is rather insignificant for the induction of potent CTL responses by heterologous prime-boost strategies.

The in vivo effect of a heterologous prime-boost protocol with rSFV and virosomes on the outgrowth of a tumor

Although heterologous prime-boost protocols with rSFV and virosomes do not result in higher cytolytic activity towards tumor cells *in vitro*, the capacity to induce higher initial precursor frequencies of specific $CD8^+$ T lymphocytes may yet make them more effective *in vivo*. To test this hypothesis, a tumor treatment experiment was performed. Previous experiments have shown that a homologous prime-boost strategy with $5x10^6$ SFV-enhE6,7 starting on day 7 after tumor inoculation can

fully inhibit tumor outgrowth in mice, while homologous prime-boosting starting from day 14 or day 17 onwards delays tumor growth but does not convey full protection[25] (Figure 4A). To be able to detect a possibly improved anti-tumor response after a heterologous prime-boost immunization, it was decided to start immunizing on day 14 after tumor inoculation and use a 5-fold lower dose. Mice were inoculated with an HPV-16 expressing tumor and subsequently immunized from day 14 after tumor inoculation onwards. The mice were either treated with three consecutive injections of $1x10^6$ SFV-enhE6,7, prime immunized with SFV-enhE6,7 and heterologously booster immunized twice with E7-virosomes, or prime immunized with E7-virosomes and booster immunized twice with SFV-enhE6,7. The booster immunizations were given one week after the previous immunization. As a negative control, a group of mice was injected 3 times with buffer. All immunization protocols resulted in delayed tumor growth compared to the buffer

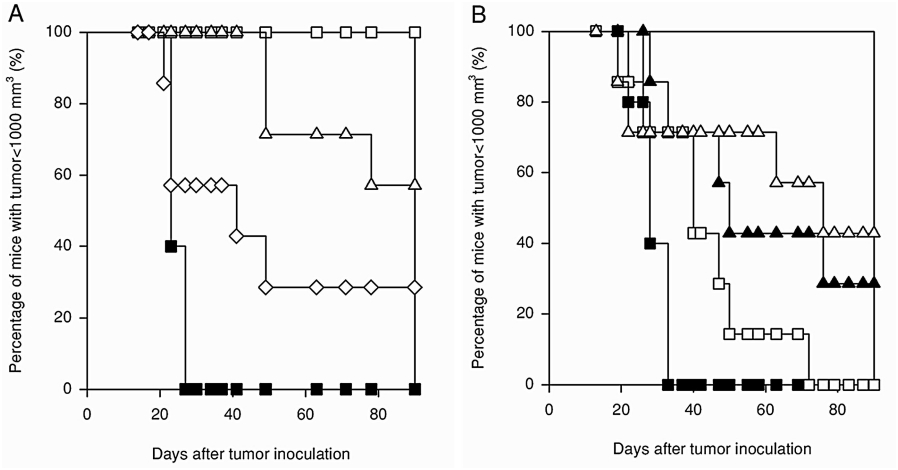

Figure 4: The effect of a heterologous prime-boost immunization protocol on the outgrowth of an HPV16 E6E7 expressing tumor.
A. In order to select a homologous immunization protocol that induces suboptimal tumor protection, mice were inoculated s.c. in the neck with $2x10^4$ TC-1 cells and treated with three weekly immunizations with $5x10^6$ SFV-enhE6,7 starting on day 7 (open squares, n=7), day 14 (open triangles, n=7), or day 17 (open diamonds, n=7) after tumor inoculation. A control group that received three buffer injections was also included (black squares, n=10). Tumor growth was measured twice weekly by palpation from day 14 onwards.
B. The effect of heterologous prime-boosting was determined by measuring tumor growth in mice prime immunized i.m. with 50 nmol E7-virosomes on day 14 after tumor inoculation and booster immunized i.m. with 10^6 SFV-enhE6,7 on days 21 and 28 (open squares, n=7), prime immunized i.m. with 10^6 SFV-enhE6,7 and booster immunized i.m. with 50 nmol E7-virosomes (open triangles, n=7), or immunized thrice according to a homologous protocol with 10^6 SFV-enhE6,7 (black triangles, n=7). A control group that received three buffer injections was also included (black squares, n=5).

control group. However, no clear difference in outcome was observed between the group immunized with SFV-enhE6,7 alone and the groups immunized according to the heterologous prime-boost immunization protocols. Ninety days after tumor inoculation 2 out of 7 mice were tumor free in the homologous prime-boost immunization group. In the heterologous prime-boost group, prime-immunized with SFV-enhE6,7 and booster-immunized with E7-virosomes, 3 out of 7 mice remained tumor free and in the other heterologous prime-boost group no mice were tumor free (Figure 4B). This experiment further demonstrates that despite the initial higher frequencies of epitope-specific precursor CTL a heterologous prime-boost immunization with rSFV and virosomes does not induce a significantly (log-rank test) stronger or more effective anti-tumor immunity.

DISCUSSION

The present study demonstrates that a heterologous prime-boost immunization protocol with SFV-enhE6,7 and E7-virosomes results in substantially higher numbers of antigen-specific pCTL than the most potent of the tested homologous protocols (immunization with SFV-enhE6,7). Co-induction of SFV-specific immunity during the virosome prime of a heterologous virosome-rSFV protocol by addition of SFV-LacZ to the E7-virosomes only slightly reduces the induction of E7-specific pCTL. Indeed, the frequency of these pCTL remained twice as high as the frequency induced by two immunizations with SFV-enhE6,7. A homologous immunization protocol with SFV-enhE6,7, as well as heterologous prime-boosting, induces CTL that can readily expand upon *in vitro* antigen-specific stimulation. Both protocols induce similar cytolytic activity towards E6/E7-expressing cells *in vitro*, as determined by ^{51}Cr release assay, and both induce the same high level of anti-tumor immunity *in vivo*. Thus, a homologous prime-boost protocol with rSFV induces equally high anti-tumor activity as a heterologous protocol with rSFV and virosomes, despite the induction of higher pCTL frequencies by heterologous prime-boosting. We furthermore demonstrate that heterologous prime-boosting strongly enhances CTL induction compared to a homologous virosome-based immunization protocol.

The induction of higher numbers of antigen-specific CTL by the heterologous prime-boost immunization protocols is in concordance with the notion that heterologous boosting focuses the response on a single or a few immunodominant epitopes shared by both immunizations and therefore results in higher numbers of target antigen-specific CTL[1;3]. La Gruta *et al*[44] showed that the immunodominance of an epitope is determined by the frequency of precursor CTL (pCTL) specific for

that epitope and the antigenic availability of that epitope. In heterologous prime-boosting with E7-virosomes and SFV-enhE6,7, the prime immunization would induce responses specific for the vector or delivery system as well as E7-specific responses. During the booster immunization, the frequency of E7-specific $CD8^+$ T lymphocytes is then expected to be much higher than the frequency of naïve $CD8^+$ T lympocytes specific for the system used in the booster immunization. Furthermore, the booster does not share the antigens of the priming vector or delivery system. Therefore, a heterologous booster would exclusively boost the E7-specific response, focusing the immune system on the target antigen and establishing E7 as the sole immunodominant antigen.

Why a virosome immunization followed by an rSFV booster results in twice the number of E7-specific CTL compared to an immunization in the reverse order is not quite clear. It may be due to the composition or intrinsic qualities of the vector or delivery system[7;10]. Conceivably, an SFV-enhE6,7 prime might disperse the focus of the immune system on two antigens; E6 and E7, whereas a prime immunization with E7-virosomes solely primes an E7-specific response. When only E7-specific responses are primed, the heterologous booster immunization may further focus the immune system and selectively boost the E7-specific CTL. That the sequence of immunizations may be crucial for the induction of high frequencies of specific precursor CTL has also been noted for other heterologous prime-boost protocols. For example, it has been found that recombinant vaccinia virus is particularly efficient in boosting immune responses primed by recombinant Influenza virus[45], recombinant fowl pox virus[2], or a protein antigen[34], whereas immunizations in the reverse order did not result in stronger immune responses in these studies. Ali S *et al*[46] have shown that a prime immunization with retrovirally transduced DC induces inherent immune-regulating mechanisms such as regulatory $CD4^+$ T cells that suppress heterologous boosting with an adenoviral vector. The higher pCTL level attained with a heterologous sequence of the virosome-prime and SFV-boost compared to vise versa might likewise be due to different levels of regulatory T cell induction by virosomes and SFV immunizations. Studies to investigate the induction of these cells by virosomes and rSFV are ongoing.

The most important observation of this study was the lack of correlation between initially induced pCTL frequencies and functional activity towards target cells. CTL induced by a heterologous immunization protocol did not expand better than CTL induced by a homologous protocol with rSFV. Furthermore, heterologous prime-boosting did not result in better specific cytolysis of tumor cells *in vitro* or *in vivo*. As *in vitro* cytolysis was determined after 5 or 7 days of restimulation, it is most likely partly dependent on the proliferating capacity of the splenocytes,

which is the same after either immunization protocol. Yet, in an article by Estcourt *et al*[5], a heterologous prime-boost protocol has been described to induce CTL with higher avidity which are expected to result in superior specific cytolysis both *in vitro* as well as *in vivo*. As reviewed by Couli PG *et al*[47], the lack of correlation between T cell numbers and cytolytic activity towards target cells could be due to inappropriate co-stimulation, or suboptimal concentrations of soluble factors such as IL-10 and IL-2 during priming of the response.

A lack of correlation between T cell numbers and cytolytic activity has also been described by others. Upon immunization of melanoma patients, distinct populations of specific $CD8^+$ T lymphocytes were identified that displayed quiescent phenotypes and lacked cytotoxic potential[48]. Rubio *et al*[49] found that tetramer staining is not directly correlated with cytolysis. Conversely, tumor infiltrating lymphocytes can consist of highly cytolytic cells that, however, do not bind tetramers[50]. Clearly, the number of antigen-specific T lymphocytes induced by a heterologous prime-boost strategy is not a good measure for the efficacy of the immunization protocol. Yet, based on studies that solely focused on analysis of the number of antigen-specific T lymphocytes, either by tetramer staining or by ELISPOT, several authors concluded that heterologous prime-boosting induces more potent immune responses than homologous immunization protocols[13;15;51]. There have also been a number of other studies, often employing a DNA prime followed by a booster immunization with a viral vector, that do show the induction of high numbers of specific T lymphocytes as well as strong functional immune responses against infectious diseases or cancer (including HPV-induced cervical cancer) by heterologous prime-boost strategies[4;11;16;18;52;53].

Evasion of vector-specific immunity, induced by the prime immunization, is often considered the most important mechanism by which heterologous prime-boost immunization protocols induce such strong immune responses[4;10]. The distinct boosting vector is thought not to be hampered by vector-specific immune responses, elicited against the priming vector, that may neutralize the vector or kill infected cells during a homologous booster[54-61]. Our finding that the addition of SFV-LacZ to the E7-virosomes prime immunization of our most potent heterologous prime-boost protocol reduces the initial number of E7-specific pCTL indicates that vector-specific immunity indeed has an effect on the booster. This effect was absent in mice primed with virosomes and boosted with rSFV, which implies that the induction of high frequencies of specific pCTL by a heterologous protocol may indeed be partly due to evasion of SFV-specific immunity. On the other hand, at the level of cytolytic activity, our results indicate that incorporation of irrelevant rSFV in the virosome prime does not hamper an SFV-enhE6,7 booster

immunization. Thus, even though the initial induction of pCTL is reduced by vector-specific immunity, evasion of vector-specific does not play a significant role in the induction of functional CTL by a heterologous booster. This is in concordance with our previous findings that SFV-specific responses do not inhibit CTL induction by SFV-enhE6,7 when these SFV-specific responses were primed in the presence of the relevant target antigen (E7) by admixing E7 protein with SFV-LacZ in the priming immunization [de Mare, manuscript in preparation].

The data presented here and in other studies[62] [de Mare, manuscript in preparation] imply that rSFV is a very potent vector in homologous prime-boost strategies and does not need heterologous priming or boosting. This is a deviation from previous heterologous prime-boost studies with rSFV[63-65], in which it was concluded that rSFV is an attractive vector for heterologous prime-boosting. However, these studies only showed the induction of increased numbers of specific T lymphocytes and stronger proliferative responses, but did not test the cytolytic potential of the induced responses. Overall, we conclude that heterologous prime-boost immunization strategies with rSFV and virosomes may result in higher numbers of specific CTL than homologous strategies with these systems. Furthermore, these higher specific CTL frequencies are mainly due to the combined intrinsic qualities of the used vector or delivery systems as well as to evasion of immunity specific for these systems. Importantly, however, our data indicate that the higher numbers of specific T lymphocytes, induced by a heterologous prime-boost immunization protocol, do not necessarily correlate with improved cytolytic activity towards target cells.

Acknowledgements

We wish to thank Solvay Pharmaceuticals for generous supplies of Influenza virus. This research was supported by the Dutch Cancer Society (RUG-2001-2361).

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CHAPTER 9

General discussion and future perspectives

The aim of the studies described in this thesis was to develop a therapeutic immunization strategy against cervical cancer and premalignant cervical disease, based on the use of a recombinant alphavirus vector system, i.e. Semliki Forest virus (SFV), the general properties of which are reviewed in **Chapter 2**. The results presented in **Chapters 3 and 4** illustrate the exquisite potency of recombinant SFV (rSFV), expressing HPV E6 and E7, to induce robust and long-lasting cell-mediated immune responses and anti-tumor effects in mice. Furthermore, the rSFV vector has the ability to turn an immune-compromised state toward immune-activation in an immune-tolerant HPV transgenic mouse model, as E7-specific cytotoxic T-lymphocyte (CTL) activity could be induced in these tolerant mice (**Chapter 5**). In addition, we showed that systemic addition of SFV encoding IL-12, known for its immune-activating and anti-tumor activity, improves the induction of antigen specific CTL activity and anti-tumor responses upon immunization with SFV-enhE6,7 (**Chapter 6**). In **Chapter 7** we demonstrate that the SFV vector system is significantly more immunotherapeutic than an adenoviral vector system. Finally, we performed a heterologous prime-boost study involving rSFV and E7 protein-containing virosomes, to further improve antigen-specific CD8+ T cell responses (**Chapter 8**).

On the basis of the results described in this thesis, it would appear that the rSFV-based therapeutic immunization strategy is ready for clinical evaluation. Clearly, the ultimate goal of such clinical studies is the implementation of therapeutic vaccination as a new control measure in the combat against cervical cancer. However, before discussing the issues involved in the potential introduction of rSFV-based therapeutic immunization against cervical neoplasia, we will first briefly reiterate other control strategies that have been implemented before.

CONTROL MEASURES AGAINST CERVICAL CANCER: A BRIEF HISTORIC OVERVIEW

While in former days cervical cancer used to be a major cause of death among women worldwide, several developments in the last century have resulted in a considerable reduction of the incidence of the disease, at least in the developed countries (Figure 1). In 1941 Georgious Papanicolaou introduced a multichromatic histological staining technique, which detects premalignant and malignant epithelial cells in cervical scrapings.[1] The implementation of this so-called "Pap-smear" marked the beginning of preventive measures against cervical cancer. Since that time, the incidence of cervical cancer has gone down by up to 75% in countries with a screening program in place based on this technique.[2] Although

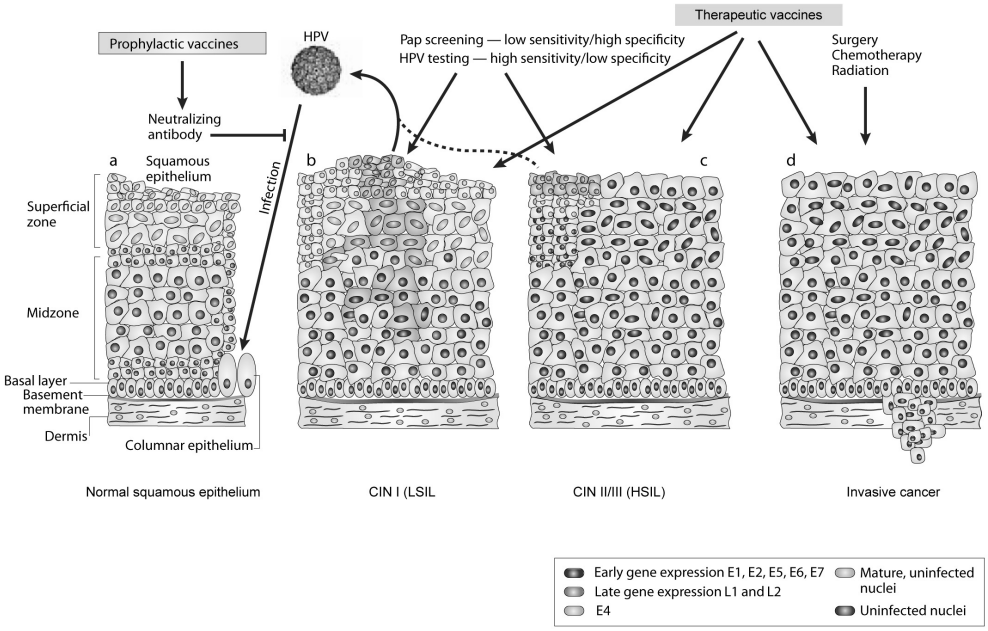

Figure 1. Schematic overview of the possible interventions against cervical intraepithelial neoplasia and invasive cancer induced by HPV.
(a) Normal squamous epithelium differentiates as shown. HPV is able to infect the basal cells of the epithelium. The transformation zone is the most common site for the development of cervical cancer. Prophylactic vaccines aim at neutralizing the virus and prevent HPV infection. (b) After HPV infection, the viral genome becomes established in the basal cells as an episome , and progeny virions are generated. In these basal layers of stratified epithelium, viral early proteins (E1, E2, E5, E6 and E7) are produced in undifferentiated keratinocytes. Infected daughter cells then begin to migrate up and differentiate in the outer layers of the epithelium. In these outer layers late proteins (L1 and L2) and early protein (E4) are produced and capsids are formed. Subsequently virions are shed into the genital tract within desquamated epithelial cells. (c) A significant fraction of high-risk HPV infections progress to high-grade lesions. In high-grade lesions the viral DNA is integrated into the host cell chromosome. Pap screening and HPV tests can be used to detect CIN lesions. CIN II/III lesions (HSIL) are effectively treated by loop electrosurgical excision (LEEP). HPV-therapeutic vaccines aim at inducing regression of established HPV infection and its (pre)malignant lesions. These vaccines will be an excellent alternative for the current treatment of CIN II/III lesions and early invasive cervical cancer. (d) The progression of untreated CIN lesions to micro-invasive and invasive cervical cancer. These cancers can be treated with surgery, chemotherapy or radiotherapy. (Reproduced, with permission from Macmillan Publishers Ltd, from Roden *et al.* Nat Rev Cancer 2006; 6 (10): 753-63)

the introduction of the Pap-smear is a major milestone in the reduction of the incidence of cervical cancer, this screening test also has limitations. First, the Pap-smear test has an average sensitivity of 51% and an average specificity of 98%. In addition, the test is associated with significant false-positive and false-negative results.[3-5] Up to a quarter of cervical cytology specimens observed as "normal", exhibit abnormalities when reviewed a second time. These false-negative results will leave cervical neoplasia undetected, leading to a delay in diagnosis and treatment.[6-8] Conversely, false-positive results, occurring in up to 14% of all cervical smears, may lead to unnecessary invasive interventions and anxiety with the patient. [9]

In 1991 Zur Hausen discovered a link between cervical cancer and the Human Papillomavirus (HPV).[10] Further extensive epidemiologic studies on the subject confirmed that persistent infection with specific "high-risk" types of HPV is essential for the development of cervical cancer.[11] To date, 15 HPV types are considered to be high-risk. The two main high-risk HPVs, types 16 and 18, are the cause of about 70% of the total number of cases of invasive cervical cancer.[12] The discovery of Zur Hausen has facilitated the development of new strategies towards prevention and treatment of cervical cancer. It has also led to the development of non-cytology-based cervical cancer screening tests.

Recently, in addition to the Pap-smear screening test, sensitive molecular tests to detect HPV DNA in cervical epithelial cells have been developed. It is now clear that HPV DNA testing is more sensitive than Pap-smears in detecting high-grade cervical intraepithelial neoplasia (CIN).[13] However, detection of high-risk HPV DNA does not prove that the HPV infection induced the formation of (pre)malignant cervical epithelial cells, since the majority of HPV infections will be transient without cytological changes. Therefore, these tests are somewhat less specific compared to conservative Pap-smear screening, which detects solely premalignant and malignant epithelial cells in cervical scrapings. Possibly, by starting the screening with HPV DNA testing followed by Pap-smears only among the HPV-positive women, the sensitivity of these diagnostic procedures for high-grade CIN lesions could be increased in comparison to conventional cytology. The use of HPV DNA testing to improve the preventive screening programs, based on Pap-smears, is currently under investigation.[14]

Despite the above efforts to reduce its incidence, cervical cancer remains the second most common cancer among women worldwide. Approximately half of the women who have acquired malignant cervical lesions will die from the disease. Over 80% of these cases occur in countries where neither population-based routine screening nor optimal treatment is available. However, also in countries where

excellent screening possibilities are in place, substantial numbers of women are still diagnosed with cervical cancer annually. One of the reasons is non-compliance with screening programs, which may be based on anxiety or embarrassment to undergo a vaginal examination. For example, half of the patients with invasive cervical cancer did not participate in the screening program.[15;16] Therefore, ongoing research to further improve prevention and treatment of (pre)malignant cervical disease is essential.

Since, as indicated above, persistent infection with a high-risk HPV is a requirement for the initiation of cervical cancer, ultimately vaccination may well be the most effective mechanism to prevent HPV infection and HPV-associated disease. Accordingly, the latest major development in reducing the incidence of cervical cancer is the recent licensure of two prophylactic HPV vaccines. As of June 2006, a prophylactic HPV vaccine developed by Merck is available under the tradename "Gardasil®". This vaccine is a quadrivalent HPV vaccine, based on virus-like particles (VLP), protective against both cervical cancer caused by HPV types 16 or 18 and external genital lesions caused by HPV types 6 or 11. Subsequently, in 2007, a similar vaccine developed by GlaxoSmithKline (GSK), called "Cervarix®", was approved in Australia and Europe. Cervarix® is a bivalent vaccine, also based on VLPs, targeting HPV types 16 and 18. In fully vaccinated women, both vaccines induce full protection from cervical dysplasia associated with the HPV types included in the vaccine and an almost 100% protection against confirmed infection with these HPV types. [17-23]

Although the introduction of prophylactic HPV vaccines represents a major step forward in the combat against cervical cancer, clearly the available vaccines only protect the high-risk types 16 and 18. Therefore, around one third of the cervical cancers will continue to develop despite the anticipated extensive implementation of prophylactic immunization against HPV. Moreover, these prophylactic HPV vaccines will not be beneficial for those women already infected with HPV, since prophylactic vaccines have no therapeutic potential.[24;25] A therapeutic vaccine against cervical neoplasia would therefore represent an additional important control measure aimed at a further reduction of the impact of cervical cancer, at least for several decades onwards.

THERAPEUTIC HPV-SPECIFIC VACCINES

Therapeutic HPV-vaccines aim at inducing regression of established persistent HPV infections and (pre)malignant lesions of the cervix. To induce regression of tumors, vaccines need to elicit a cell-mediated cytotoxic T-lymphocyte (CTL)

response, leading to elimination of (pre)malignant cells. In HPV-induced lesions, the expression of the oncoproteins of HPV, E6 and E7, occurs in cells in which the viral genome has integrated into the cellular DNA. The E6 and E7 proteins allow virus-infected cells to escape apoptosis and cell cycle arrest. Constitutive expression of these proteins is therefore a prerequiste for the maintenance of the transformed phenotype of (pre)malignant cells, making E6 and E7 in fact tumor-specific antigens in (pre)malignant cervical lesions. As a consequence, E6 and E7 represent suitable targets for an HPV-specific therapeutic vaccine.[26;27]

Requirements for a therapeutic vaccine against cervical neoplasia

To obtain an effective therapeutic vaccine against cervical cancer and its precursor lesions several barriers have to be overcome. A major challenge for an effective therapeutic vaccine against cervical neoplasia is the immunocompromised state of many of the patients involved. It has been suggested that cervical cancer patients have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens.[28-34] In addition, HPV-infected cells use various immune-escape mechanisms to evade host immunity.[35-39] These immune evasion mechanisms may lead to local immune nonresponsiveness. As a consequence, HPV antigen-specific effector cells may either not be recruited to the infected area, or their activity may be downregulated.[38] This puts high demands on potential therapeutic vaccines, since the vaccination needs to overcome this immune tolerance in order to be effective.

Another requirement for a therapeutic vaccine against cervical cancer is the need for a high level of biosafety. There are concerns about the safety of several vaccine candidates, since the targets of these therapeutic vaccines are the E6 and E7 oncoproteins of the high-risk HPV type involved. For example, the use of vaccines based on DNA or certain viral vectors, harbors the potential risk of integration of the E6 and/or E7 genetic material into the host cell genome. As a consequence, this may lead to malignant transformation of the cells hit by the vaccine. On the other hand, when peptide/protein-based vaccines or vaccines using vectors based on viruses, where no DNA intermediates are being formed, this risk of integration is not an issue. When the therapeutic vaccine is based on the use of viral vectors, another general concern represents the possible formation of infectious virus particles. Therefore, depending on the approach of vaccination, measures have to be taken to guarantee safety.

Current therapeutic HPV-specific vaccine candidates

Several candidate therapeutic HPV vaccines have been evaluated in Phase I and II

clinical trials. To put rSFV as a potential therapeutic vaccine in perspective, we will first briefly discuss the pros and the cons of the available approaches. Presently, the three main forms of HPV therapeutic vaccines are peptide/protein-, DNA-, and viral vector-based vaccines.

1. Peptide/protein-based vaccines

The major advantage of peptide and protein-based vaccines is that in principle they offer a high level of safety. HPV-specific therapeutic vaccines based on peptides and proteins have been shown to be well-tolerated in humans, without the occurrence of significant adverse effects. Another advantage of peptide- or protein-based vaccines over other approaches is their relatively low cost and ease of production. Yet, an important disadvantage is that these vaccines in general are poor inducers of CTL activity. In most cases, the antigen involved is not actively delivered to the cytosol of antigen-presenting cells (APCs), and as a consequence there is no presentation in the context of MHC class I via the endogenous route. These peptide/protein-based vaccines will, therefore, normally be dependent on a process called cross-presentation for delivery of the antigen to the MHC class I processing and presentation pathway. Although this process of cross-presentation does induce CTL activity, it appears to be much less efficient than CTL induction via the direct priming of APC. (Jan, zie ook opmerking van Toos in de kantlijn) [40;41]

Most preclinical model studies and clinical evaluation of therapeutic HPV immunization strategies are based on the use of peptide or protein vaccines.[42-51] So far, the majority of clinical trials using these vaccines have shown limited efficacy in eradicating established HPV-induced malignancies in humans. The weak induction of cell-mediated immunity by these vaccines may present a major problem, since, as explained before, strong CTL response will likely be necessary to induce an effective anti-tumor response in cervical cancer patients. Efforts have been made to enhance the induction of CTL responses upon immunization with peptide and protein vaccines through the use of adjuvants or specific antigen-delivery strategies.

Recently, Zwaveling *et al.*[52] showed that vaccination with long peptides resulted in more potent CTL responses than vaccination with a peptide corresponding to the exact minimal CTL epitope involved. Longer peptides allow presentation of more CTL epitopes and T-helper epitopes, resulting in enhanced immunogenicity. This may result in a higher efficacy in cervical cancer patients. Future clinical trials will have to show the potency of this approach among patients with HPV-induced cervical lesions.

The use of virosomes as delivery system represents a promising strategy to make peptide and protein vaccines more effective. Recently, it was shown that influenza virosomes are potent inducers of target antigen-specific CTL responses. Virosomes actively deliver the antigen to the cytosol of APC, leading to presentation of the antigenic peptides in the context of MHC class I via the endogenous route.[42;53] As a result strong CTL activity and anti-tumor responses can be induced with low doses of antigen.[54-56] In addition to their function as delivery vehicle for introduction of the antigen into the cell cytosol, virosomes may also act as an immune adjuvant.[57] Virosome-based vaccines are already approved for use in humans. Given these properties, virosomes containing E6 and/or E7 protein antigen represent an attractive therapeutic vaccine candidate. In **Chapter 8** we describe the use of E7-containing virosomes, in conjunction with rSFV-enhE6,7, in a prime-boost immunization regimen.

2. DNA-based vaccines

The main advantages of DNA-based vaccines are their purity, ease of preparation and stability. In addition, DNA vaccines do not elicit antibodies against the DNA itself, and thus can be administered repeatedly. Furthermore, DNA vaccines may prime class I MHC-restricted CD8+ T cell responses, which may be advantageous compared with conventional protein-based vaccines.[58-60] Indeed, it has been demonstrated that, upon immunization with DNA-based vaccines, not only humoral but also cell-mediated immune responses are induced against HPV-antigens.[61] Immunization with DNA vaccines results in expression of antigen in the context of MHC molecules over a longer period of time compared with peptide or protein vaccines. However, in the case of HPV-specific therapeutic vaccines, the prolonged persistence of the E6 and/or E7 DNA also represents a safety concern. Another major drawback is that naked DNA vaccines are comparatively weakly immunogenic. Although these DNA-based vaccines are able to induce a cell-mediated immune-response, they only result in low numbers of precursor CTLs.

Various strategies have been developed to enhance DNA vaccine immunogenicity.[62-66] As with peptide/protein-based vaccines, a strategy to improve the immunogenicity of HPV DNA vaccines is by encapsulating the DNA into a delivery system. An interesting example is the use of encapsulated plasmid DNA-encoding fragments derived from E6 and E7 of HPV16 and HPV18 in biodegradable particles (ZYC101a), which enhance delivery to APCs. Recently, it was reported that the use of ZYC101a resulted in a significantly higher rate of CIN II/III resolution in the treated groups under the age of 25 years. However, this effect

was less pronounced in the group older than 25 years of age. This may be partly explained by the fact that younger women have been exposed to the HPV for shorter periods of time. Therefore, the HPV-transformed cells may have suffered fewer virus-induced changes, making them more sensitive to immunotherapy.[62] Although promising, further improvements in the potency of ZYC101a are still needed in order to make this strategy sufficiently strong to treat patients with CIN II/III lesions.

Another promising strategy to further enhance the potency of DNA vaccines is the use of so-called particle-mediated epidermal delivery (PMED). This approach efficiently delivers gold particles coated with DNA plasmids encoding vaccine antigens into the epidermal layer of the skin.[67;68] This system enables DNA to directly transfect professional APCs, allowing antigen presentation via both MHC class I and II molecules.[69] Clinical trials have demonstrated that particle-mediated DNA immunization efficiently induces both antibody and T cell responses, requiring a significantly lower dose of DNA compared to inoculation of DNA with a needle and syringe.[70-73] This ability of PMED to effectively induce Th and CTL responses in humans suggests that this strategy might also be useful in the context of the potential development of a DNA-based therapeutic HPV-specific vaccine.

However, despite the fact that there are promising developments, taken together, the weak potency of DNA vaccines in inducing cellular immune responses, the safety concerns related to the use of DNA vaccines, particularly in the context of HPV vaccination, and the so far limited clinical response in patients with CIN lesions, make DNA-based vaccines at this point less attractive for therapeutic vaccination purposes against cervical cancer.

3. Vaccines based on viral vectors

Viral vectors, mimicking real-virus infection, in general are very potent immunogens. Indeed, a major advantage of viral vectors over the approaches mentioned above is their ability to induce massive cellular immune activation. Upon infection with a recombinant viral vector expressing an antigen of interest, the antigen is synthesized endogenously within the cytosol of the target cell. Thus, CTL epitopes are processed and presented in the context of both MHC class I and class II in a natural manner, resulting in the induction of both humoral and cellular immune responses to the antigen. In addition, since these vaccines mimic a real-virus infection, also the innate immune response may be activated resulting in a strong enhancement of the induced immune response. Nonetheless, despite these advantages, there are several drawbacks, including potential safety issues and pre-existing immunity against the virus that is used as a vector, which are

dependent on the type of virus used. These issues will be further discussed for DNA viruses versus RNA viruses.

Vectors based on DNA viruses

The two main vectors based on DNA viruses which have been used extensively in preclinical and clinical studies against cervical cancer, are recombinant vaccinia virus and adenovirus.

Vaccinia vectors are widely used in vaccines (vaccinia/smallpox vaccine). These vectors can be relatively easily manufactured. Vaccinia vectors have a stable dsDNA genome with a large cloning capacity. Phase I and II clinical trials using recombinant vaccinia virus encoding HPV16 and 18 E6/E7 (also called TA-HPV) demonstrated that it was well-tolerated and indicated that some patients with CIN lesions or advanced cervical cancer, developed T-cell immune responses upon vaccination.[74-76] TA-HPV has also been used in the treatment of high-grade HPV16-positive vulval intraepithelial neoplasia (VIN). Most patients who received a single dose of the vaccine demonstrated HPV16-specific immune responses. However, no complete correlation between immunological and clinical responses could be defined.[77]

Vectors based on recombinant adenovirus type 5 have been evaluated for a variety of diseases ranging from infectious diseases to cancer.[78-81] The vectors based on recombinant adenovirus are rendered replication-defective by mutations and deletions. Adenoviral vectors infect cells and deliver their genomes to the nuclei of the target cells, resulting in sustained presentation of the antigens. With a cloning capacity of approximately 8 kb, these vectors allow insertion of relatively large genes. They can be prepared easily in high titers and can efficiently transduce a wide range of cell types.[82] Recombinant adenoviruses have been shown to elicit strong humoral and cell-mediated immune responses. Several preclinical studies in mice using adenoviral vectors expressing HPV-16 E6 and/or E7 showed enhanced antigen-specific CD8+ and CD4+ T-cell immune responses and anti-tumor effects.[83-85] To date, no clinical trials have been performed using this vector as a therapeutic vaccine against cervical cancer.

In this thesis, as a prelude to future clinical evaluation of rSFV, the efficacy of rSFV was compared with that of a recombinant adenoviral vector, because adenoviral vectors, in contrast to rSFV, have been used extensively before in clinical trials. In **Chapter 7** we show that the SFV vector system proved significantly more immunotherapeutic than the adenoviral vector system. We hypothesize, based on the results, that following immunization with recombinant adenovirus (rAd), the induction of CTL memory is disturbed by at least two mechanisms. Firstly, strong

anti-vector responses induced after immunization with recombinant adenovirus may prohibit the effect of booster immunizations necessary for efficient memory CTL induction.[86] Secondly, the prolonged expression of antigen with recombinant adenovirus may lead to the induction of effector T cells, which fail to acquire the key properties of memory cells.[87;88] For rSFV these issues will be discussed further below.

For both recombinant vaccinia and adenovirus vectors, there are concerns about pre-existing immunity. As mentioned above, vaccinia virus has been used extensively as a smallpox vaccine, resulting in anti-vector immunity in vaccinated people. Also worldwide, there is a high seroprevalence against adenovirus, in particular serotype 5.[89] It has been demonstrated that this anti-vector immunity essentially abrogates the ability of recombinant adenovirus serotype 5 to serve both as a priming or booster vector.[90] Therefore, adenovirus vectors derived from strains that have not circulated widely in the human populations are under investigation as vaccine vectors. Another way to evade anti-vector immunity involves the use of heterologous prime-boost strategies.[91;92] However, a recent clinical study using a prime-boost strategy with recombinant vaccinia virus and a DNA vaccine could not show a simple relationship between induction of systemic HPV16-specific immunity and clinical outcome.[93]

Another concern related to the use of vaccinia virus or adenovirus vectors as a HPV-specific therapeutic vaccine, relates to the potential integration of the E6 and E7 DNA into the genome of the vector-infected cells. Since vaccinia and adenoviruses are DNA viruses, the replication takes place in the nucleus of the cells, giving rise to the potential possibility of nuclear integration, and hence transformation of the infected cells. Therefore, the targets used in these vaccines should be modified, eliminating the risk for malignant transforming capacity, and at the same time preserve the immunogenicity of the antigens. In addition, for vaccinia there is the concern about potential dissemination of vaccinia to immunodeficient individuals.[94-96] Another major safety concern with adenovirus vector is the adverse inflammatory response that may be generated by high doses of the vector; this inflammatory response has led to the death of a patient in an adenovirus clinical trial.[96-98] Recently, an adenovirus-based HIV vaccine Phase II trial demonstrated to be ineffective at either preventing infection of individuals not previously infected with HIV or at reducing viral loads in those individuals who became infected with HIV during the trial. Moreover, these results indicated that among individuals with pre-existing immunity to adenovirus, there were more infections in vaccine-recipients than in study participants who received placebo. The reasons for this result are still being studied.[99]

In conclusion, although the described viral vectors are available for clinical use and have shown promising results, the concerns about the safety, pre-existing immunity, and the potential unsuitability for homologous prime-boost immunization protocols make them possibly less attractive candidates for a therapeutic vaccine against cervical cancer.

Vectors based on RNA viruses

Several RNA virus vaccines are being used or explored in clinical and preclinical settings against a variety of diseases. Among the RNA viruses used are Vesicular Stomatitis Virus (VSV), measles virus, poliovirus, and alphaviruses.[100] A major advantage of the use of vectors based on RNA viruses over vectors based on DNA viruses, as a HPV-specific therapeutic vaccine, is that the safety concern about integration is not an issue. This is because the replication of RNA viruses takes place in the cytoplasm without formation of DNA-intermediates. Particularly, vectors derived from alphaviruses (i.e. Sindbis virus, Semliki forest virus, or Venezuelan equine encephalitis virus) are gaining increased interest for their high transfection potency and strong immunogenicity.

Alphaviruses are zoonotic and only cause infrequent epidemics among humans in certain geographical regions. Therefore, in contrast to the vectors based on major human pathogens, pre-existing immunity against alphaviruses is rare and is unlikely to present a problem. Recombinant alphaviruses induce high-level expression of encoded foreign proteins. After 48-72h of protein expression, infected cells die by apoptosis resulting in apoptotic bodies, containing high levels of the transgene protein, which may be very effective in the induction of immune responses via so-called cross-priming.[101;102] Thus, these vectors efficiently induce both cellular and humoral immune responses to the expressed antigen. In **Chapter 2** an overview is given of the recombinant alphaviruses used as vectors for anti-tumor and anti-microbial immunotherapy.

Besides the shared characteristics of the various alphaviruses, there is a difference with respect to the delivery of encoded protein antigens to DCs for MHC class I and MHC class II processing and presentation. Venezuelan equine encephalitits virus and Sindbis virus directly transfect murine DCs (direct priming), while rSFV is dependent for the presentation of antigens on a process of cross-priming.[101;103-106] Despite the difference in tropism for DCs, immune responses elicited upon immunization with these different alphavirus vectors are in general comparably efficient. Other preclinical studies using alphavirus vectors as therapeutic HPV specific vaccines also show promising results, like the results described in this thesis. Upon immunization with these vectors strong anti-tumor

activity and high antigen-specific CTL responses are induced.[107-110] Since this thesis mainly focuses on the immunotherapeutic effect of a recombinant SFV viral vector, the remainder of the discussion will be confined to rSFV.

RECOMBINANT SFV AS A THERAPEUTIC VACCINE

A potent and safe system for the induction of CTL and anti-tumor responses

The preclinical studies described in this thesis demonstrate the exquisite efficacy of therapeutic immunization based on SFV in an HPV-murine model. As described in **Chapter 3** and **Chapter 4** of this thesis, exponentially growing tumors of approximately 500 mm^3 in size were seen to completely resolve upon therapeutic immunization with SFV-enhE6,7. The magnitude of this immune and anti-tumor responses against HPV16-induced tumors is determined by the dose administered and by the route of immunization with SFV-enhE6,7. Notably, i.v. and i.m. immunizations resulted in significantly higher pCTL frequencies and superior anti-tumor responses compared with s.c. immunization (**Chapter 4**). Another important aspect of our immunization approach is the induction of a long-term memory immune response. Even half a year after immunization, mice are able to eradicate s.c. inoculated tumors and up to 340 days after immunization high levels of CTL activity can be observed (**Chapters 3 and 4**). We further demonstrated that, depending on the route of immunization, SFV-enhE6,7 has the capacity to induce HPV16 E7-specific CTL activity in immune-tolerant HPV-transgenic mice (**Chapter 5**). As mentioned previously in this discussion, cervical cancer patients have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens. Although the mechanism and kinetics of tolerance in this transgenic mouse model certainly differ from that in the human clinical situation, these observations do underline the anticipated efficacy of SFV-enhE6,7 for immunization purposes in these patients.

The high potency of rSFV can be ascribed to several features of the system. As stated earlier, the main mechanism by which immunity to a rSFV is generated is via cross-presentation of antigen by DCs that have taken up apoptotic bodies derived from rSFV-infected cells.[101;103] The apoptotic bodies contain high levels of transgene protein which are likely to be very effective in the induction of immune responses. Another important feature of rSFV is that infection of cells with SFV results in the formation of dsRNA intermediates that are known for their immunopotentiating capacity.[111] These dsRNAs can be recognized by innate immune receptors, such as Toll-like receptor 3, and trigger production of type I

IFN, while, in addition, dsRNAs induce activation and maturation of DCs.[112] For the differentiation, expansion and memory induction of antigen-specific CTLs, Th-cells and properly activated APCs are essential. Uptake of apoptotic cells transfected with rSFV will thus not only provide the specific antigen, but will also provide the required danger signal to turn immature tolerizing DCs into mature, activating DCs. In **Chapter 7** we demonstrate that the SFV vector system is significantly more immunostimulating than a recombinant adenoviral (rAd) vector. Although rAd is able to infect DCs directly, the Ad-mediated transduction of DCs is inefficient.[113] DCs activated *in vitro* by rAd are inhibited to fully mature and polarize toward a Th1-inducing phenotype, which is essential for eliciting an effective immune response.[114] Furthermore, the induction of memory T cells generated upon immunization differs with both vectors. As explained previously for rAd, the induction of memory T cells may be disturbed. In contrast, our studies suggest that the rSFV vector system possesses the ability to generate just the right level of stimulation for the induction of memory T cells.[115]

Beside its strong potency, another important feature of the SFV vector system is the high level of biosafety. rSFV are "suicide" particles in that they induce one round of infection, during which the viral RNA is self-amplified by the replicase complex, resulting in a high level of expression of the foreign gene. Foreign protein expression, however, is transient since the infected cells undergo apoptotic cell death.[116;117] As the replication of SFV is only cytoplasmic, without a DNA intermediate[118], there is no possibility of nuclear integration of the foreign gene or insertional mutagenesis. Since the structural genes of the virus are missing, new infectious progeny particles cannot be formed.

The rSFV virus is generally produced by cotransfection of cells with the recombinant RNA vector and a so-called helper RNA vector, coding for the capsid and spike proteins. Since the RNA packaging signal is absent on the helper vector, only the recombinant RNA is packaged into newly generated virus particles, which can thus induce just one round of infection. Yet, during the production of recombinant virus, there is a small but still significant chance that RNA recombination occurs with formation of infectious virus.[117] The formation of infectious virus through RNA recombination may however be reduced to insignificant levels through the use of split helper vectors in the production of the recombinant virus, virtually eliminating the chance that productive recombination occurs.[119] In fact, no replication-competent virus has been detected in rSFV preparations produced with the use of split helper RNAs. [120]

Is there a need for further improvement of the rSFV-based strategy?

In our research we have focused on optimization of the efficacy of recombinant SFV in terms of induction of effective antigen-specific CTL responses. The results presented in **Chapters 3 and 4** illustrate the exquisite potency of the system to induce robust and long-lasting cell-mediated immune responses and anti-tumor effects in mice. Also, the rSFV vector has the ability to turn an immune-compromised state toward immune-activation in immune-tolerant HPV-transgenic mice (**Chapter 5**). However, the levels of specific cytolysis induced in the transgenic mouse model were significantly lower than those induced in wild-type mice. Therefore, we investigated whether co-administration of an immuno-stimulatory adjuvant, IL-12, to SFV-enhE6,7 could further stimulate CTL induction and anti-tumor responses, in particular in immune-tolerant mice (**Chapter 6**). Although, in both wild-type and transgenic mice, the co-expression of IL12 did significantly stimulate CTL induction, unfortunately, the extent of stimulation did not suffice to generate a measurable anti-tumor response in the transgenic mice. It should be noted, however, that the immune tolerance in transgenic mice is likely to be very stringent and substantially different from that in patients with cervical neoplasia. Therefore, although the adjuvant effect of SFV-IL12 upon immunization of mice with SFV-enhE6,7 was limited, there may be still be a therapeutic benefit associated with it in the clinical setting.

Another level at which the rSFV system could potentially be further improved is that of anti-vector immunity. As discussed above, anti-vector immunity represents a major problem associated with the use of vaccines based on recombinant viruses. The main effector mechanism of vector-specific immunity is thought to be neutralization by antibodies specific for the structural proteins of the vector.[121-123] Until recently, anti-vector responses elicited by rSFV were not very well documented. In **Chapter 7** we demonstrate that repeated administration of rSFV to mice results in a significant decrease in transgene expression. It is generally thought that such reduced levels of transgene expression may eventually lead to lowered CTL induction.[121;122;124] The fact that, despite the significant decrease in transgene expression, rSFV is still capable of inducing a strong CTL response, can in part be explained by the dose of rSFV used, which was in general approximately 100-fold higher than the minimal effective dose of rSFV. As a consequence, even in the presence of significant levels of virus-neutralizing antibodies, rSFV is still capable of inducing enough transgene expression to result in a strong antigen-specific CTL activity and anti-tumor responses. These results also underline that the rSFV system is particularly well suited for use in homologous prime-boost immunization protocols, since - despite the reduced levels of transgene expression

upon repeated administration - CTL (re)activation does not appear to be hampered in wide dose range studied.

This conclusion is further supported by recent observations of De Mare *et al.*[125], who demonstrated that transgene-specific CTL induction by rSFV is not so much affected by vector-neutralizing antibodies, but rather influenced by T cell competition. T cell competition may play an important role in vector-specific immunity. During T cell competition, vector epitopes and epitopes of the target antigen are both presented on the same APC. As a consequence the immune response may focus on the antigens of the vector instead of the target antigen, resulting in a reduced target-antigen specific immune response.[126;127] Due to neutralization by vector-specific antibodies and killing of infected cells by vector-specific CTLs, most other viral vectors are unsuited for homologous prime-boost immunization protocols.[90;128-131] In contrast, the study of De Mare *et al.* demonstrates that rSFV is a very powerful vector for homologous prime-boost immunization protocols, since the reduced transgene expression due to vector-neutralizing antibodies has little effect on CTL induction by rSFV in a wide dose range, and T cell competition, possibly due to the strong immunodominance of the target antigen, does not play a role in homologous immunization protocols.

In our efforts to optimize SFV-mediated CTL responses, we also performed heterologous prime-boost immunizations, involving rSFV in conjunction with influenza virosomes carrying the HPV E7 protein antigen. This is a way to evade SFV-specific immunity and might possibly lead to higher CTL responses. It has been demonstrated that with this strategy powerful synergistic effects can be achieved, reflected in an increased number of antigen-specific T cells, selective enrichment of high-avidity T cells and increased efficacy against pathogen challenge.[132;133] Additionally, these protocols may generate improved effector memory CD8+ T cell responses.[134] Heterologous prime-boost strategies prime the immune system to a target antigen delivered by one vector and then selective boost target-specific immunity by re-administration of the antigen in the context of a second distinct vector. In **Chapter 8**, surprisingly, no difference in cytolytic activity of CTL induced by a heterologous booster in the presence or absence of SFV-specific responses was found compared to a homologous protocol with rSFV. Furthermore, both heterologous and homologous protocols induce the same high level of anti-tumor immunity in mice. These findings support our above conclusions and imply that, at the level of cytolytic activity, rSFV is not hindered by the anti-vector immunity. This underlines that rSFV can be used both as a priming and a boosting vector.

Challenges on the way toward clinical evaluation of the rSFV system

As mentioned in the first part of this discussion, the ultimate goal of our studies is to develop a therapeutic vaccine against cervical cancer and premalignant cervical disease. Besides all the advantages of rSFV, a number of major challenges on the way toward clinical application of rSFV remain. First, the production of large amounts of clinical-grade rSFV in itself may represent a challenge. To generate rSFV, cells have to be electroporated in the presence of recombinant SFV RNA and helper RNAs. Upon introduction of these RNAs into the cytoplasm of the producer cells, the RNAs will replicate and will be translated, resulting in the generation of new recombinant virus particles. Secondly, in contrast to the several other viral vector systems, rSFV has not yet been evaluated in a clinical setting. Therefore, it is difficult to predict how rSFV will perform in humans. However, the preclinical studies described in this thesis are promising and fully justify clinical trials to evaluate the potential of rSFV-based therapeutic vaccination in patients with (pre) malignant cervical lesions.

FUTURE PERSPECTIVES

In this thesis, we have focused on determining the efficacy of a therapeutic immunization strategy based on rSFV against HPV-induced cervical neoplasia. As discussed in the preceding paragraph, the described preclinical studies justify clinical trials to evaluate the efficacy of rSFV to treat patients with (pre)malignant lesions. To finalize this discussion it is important to consider the position of therapeutic vaccination in the current preventive and curative setting.

The recent introduction of HPV prophylactic vaccines

This discussion started with an overview of the efforts to reduce the incidence of cervical cancer. As mentioned, the most recent development added to the available preventive options against cervical cancer are the prophylactic HPV vaccines developed by Merck and GlaxoSmithKline (GSK), named "Gardasil®" and "Cervarix®", respectively. Clinical trials with both vaccines have shown exciting results. More than 99% seroconversion against the HPV types included in the vaccines was induced, and peak antibody titers were at least 50-fold higher than the titers detected after natural infection. In fully vaccinated women, both vaccines induced full protection from cervical dysplasia associated with the HPV types included in the vaccines and an almost 100% protection against confirmed infection by the same HPV types.[17-23] However, although these highly effective and apparently safe vaccines have great potential for reducing cervical cancer rates,

several issues remain unresolved.

First, the duration of the antibody protection beyond 5 years is unknown. Over the next several decades it will be important to monitor antibody levels and HPV infections in immunized subjects to evaluate the duration of protection and to determine whether booster vaccinations will be needed. In addition, it will be important to determine whether the prophylactic vaccines also induce an effective memory response, giving long-lasting protection even after disappearance of vaccine-induced antibodies.

A second issue is that the two vaccines protect only against two of the high-risk HPV types, which are responsible for only 70% of the cervical carcinomas. As a consequence, the vaccines do not protect against the other 30%.[135;136] These prophylactic vaccines will thus reduce, but not eliminate, the risk of cervical cancer. This implies that also in the longer term, current screening programmes will need to be maintained.

Another issue is that girls will need to be vaccinated before they become sexually active, between the age of 9 and 13 years to prevent genital HPV infection.[137] Although some countries have successful school-based programmes to deliver vaccines to adolescents, many do not. Also, parental consent will be needed, and it has been suggested that about one fifth of the parents are likely to deny consent for their children to be vaccinated.[138]

Furthermore, until now clinical trials determining the efficacy of prophylactic HPV vaccination have mainly been focused on women. Data documenting vaccine efficacy in men are limited. Although the burden of HPV-induced disease is considerably less in men compared to women, it is not insignificant.[139] Since HPV is a sexually transmitted disease, involving both men and women, future studies will have to investigate whether these vaccines are also effective in men, and should be recommended for them as well.

Finally, the vaccines are not therapeutic. Therefore, it is unlikely that these vaccines will have much impact on those already infected with HPV. Thus, even if large-scale prophylactic vaccination was implemented today, it would take decades to lower incidences of HPV-induced pre-malignant lesions and invasive cervical cancer.

The need for and the position of an HPV-specific therapeutic vaccine

So far, in the developed world, cervical cancer or preceding CIN lesions are usually detected at an early stage, as a result of intensive screening programs. Cytomorphological examination of cervical smears is the most widely applied screening method for cervical cancer and its precursors. Despite these excellent

screening possibilities, substantial numbers of women are still diagnosed with cervical cancer annually. Approximately 90% of all the CIN II and III lesions can be treated successfully with the current, mostly surgical, treatments. Yet, these treatments are often invasive and disfiguring for women and are also related with increased pregnancy-related morbidity.[140] Furthermore, the overall rate of recurrent or persistent disease is 5 to 17%.[141] Thus, ongoing research to further improve the present preventive and therapeutic options is desirable.

Therapeutic HPV vaccines will be an excellent alternative for the current treatment of CIN II and III lesions and early invasive cervical cancer. They will represent a less invasive and disfiguring treatment option for women with pre-existing HPV-associated lesions, and may prevent recurrence or persistence of the disease. In addition, a therapeutic HPV vaccine might also be beneficial for treatment of other HPV-associated non-cervical cancers.

Given that the present prophylactic HPV vaccines will not protect against the HPV types not included in the vaccines, around one third of the cervical cancers will continue to develop even if prophylactic vaccination were widely implemented. Also, prophylactic vaccines will not be effective in women already infected with HPV. Therapeutic HPV vaccines could fill in this temporal deficit of prophylactic vaccination, by attacking already established HPV infection and HPV-induced disease. At the time an effective therapeutic vaccine can be launched, together with the prophylactic vaccines, this is expected to result in significant reductions of health-care costs and a further reduction of cervical cancer incidence and death.

CONCLUDING REMARKS

The robust therapeutic immune responses elicited by rSFV-enhE6,7 vaccine candidate, the absence of pre-existing antibodies in the human population, the ability to use the vector both in priming and booster immunizations, and the high level of biosafety of the system, all position rSFV as a serious candidate for clinical evaluation as a potential treatment for patients with (pre)malignant CIN lesions and early invasive cervical cancer.

A possible hurdle to clinical application of rSFV is that this alphavirus vector system has not yet been used in a clinical setting. This may slow down procedures involved in obtaining approval for initial clinical studies. However, if promising results emerge from early trials, possible reservations with respect to these vectors will likely diminish.

Finally, we started this discussion with a survey of control measures against cervical cancer that have been implemented over the last several decades. These

control measures have been very successful and have reduced the incidence of cervical cancer substantially. However, this applies almost exclusively to the developed countries. It is important to note that over 80% of all cases of cervical cancer occur in underdeveloped and developing countries, and this proportion is expected to increase to 90% by 2020.[142] Women in these countries, who would benefit most from the vaccination, are unlikely to receive the vaccine. Apart from the technical and logistic issues involved, they simply have no access to it and they cannot afford it. Neither will these women receive a Pap smear test, again because of a lack of access and money. Therefore, clearly the most difficult challenge in the combat against cervical cancer worldwide will be to find possibilities to give girls and women in developing countries an equitable access to prophylactic, and potentially also therapeutic, vaccines as well as to screening and subsequent treatment options.

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CHAPTER 10

Summary

The aim of the studies described in this thesis was to investigate the efficacy of a therapeutic immunization strategy against cervical cancer and premalignant cervical disease. Cervical cancer is caused by persistent infection with high-risk human papillomavirus (HPV). Two of the early proteins of these high-risk HPV, E6 and E7, interact with the cell cycle regulation proteins p53 and pRb, respectively, and can cause immortalization of cells. Constitutive production of these oncoproteins is required for the maintenance of the transformed phenotype of the cells, making these proteins attractive targets for immunotherapeutic strategies against HPV-induced cervical lesions. The therapeutic immunization strategy described in this thesis is based on an alphavirus vector, i.e. Semliki Forest virus vector (SFV). Recombinant SFV expressing a fusion protein of E6 and E7 under control of a translational enhancer (SFV-enhE6,7) is investigated in several preclinical studies using a murine model for HPV-dependent carcinogenesis.

Chapter 1 presents a general introduction on cervical cancer, the etiological role of HPV infection in the induction of (pre)malignant lesions, and the current treatment options for the different stages of the disease. Next, the role of the immune system in the development of disease is described. Finally, an overview of currently available prophylactic and therapeutic immunization options, both registered and experimental, is given. Cervical cancer is the second most common cancer among women worldwide. As mentioned earlier, the etiology of cervical cancer is a persistent infection with a high-risk type HPV.[1-3] High-risk HPVs, in particular types 16, 18, 31, 33 or 45, have the capacity to transform cervical epithelial cells through integration of the open reading frames encoding the viral early proteins E6 and E7 into the host cell genome.[4;5] Since the continued production of these oncoproteins is required for the maintenance of the transformed phenotype of the cells, E6 and E7 in fact represent tumor-specific antigens in cervical carcinoma and premalignant HPV-transformed cells. As a consequence, E6 and E7 are suitable targets for immunization strategies against cervical cancer.[6]

Therapeutic immunization against virus-infected cells or tumor cells requires the induction of cytotoxic T-lymphocytes (CTLs) that can specifically recognize and lyse infected or tumor cells. For the differentiation, expansion and memory induction of antigen-specific CTLs, T-helper (Th) cells and properly activated antigen presenting cells (APCs), dendritic cells (DCs) in particular, are essential. The majority of the HPV infections are transient, not giving rise to detectable cervical lesions. However, HPVs have evolved different immune evasion mechanisms such that infected cells and (pre)malignant cells may not always be recognized effectively by the immune system.[7] Furthermore, it has been suggested that

patients with CIN lesions or cervical cancer are immunosuppressed and/or have mounted a certain degree of immunological tolerance or ignorance for the HPV-derived antigens.[8] Therefore, the challenge in tumor vaccination will be to design immunotherapeutic modalities that are potent enough to turn the balance from immune tolerance towards immune activation.

In **Chapter 2** an overview is given of the recombinant alphaviruses used as vectors for anti-tumor and anti-microbial immunotherapy. Alphaviruses are small, enveloped, positive-strand RNA viruses belonging to the family *Togaviridae*. The prototypic vectors are derived from Semliki Forest virus (SFV), Venezuelan Equine Encephalitis virus (VEE) and Sindbis virus (SIN). In the presence of helper vectors, recombinant RNA derived from these viruses generates recombinant virus particles that are limited to one round of infection. The recombinant RNA is self-replicating and transiently expresses the foreign gene(s) at high levels.

Vectors based on alphaviruses are gaining increasing interest because of their superiority over other viral vectors with respect to the induction of both humoral and cellular immune responses. Characteristics that make alphaviruses attractive candidates for development of replicon vectors for application in humans are that (i) recombinant alphaviruses induce high-level expression of encoded proteins, (ii) after 48-72h of protein expression, infected cells die by apoptosis resulting in apoptotic bodies containing high levels of the transgene protein which may be very beneficial for the induction of immune responses via cross-priming[9] (iii) recombinant alphaviruses activate both the innate and the adaptive immune system.[10;11]

Different immunization strategies against infectious diseases show that the induction of humoral responses upon immunization with alphaviral vectors varies a great deal depending on the antigen, the processing and presentation of the antigen and the immunization route. Nonetheless, in most studies, strong humoral and cellular immune responses are induced that result in (partial) protection against specific micro-organisms. Tumor vaccines based on alphaviruses are in general designed to stimulate or augment a cellular immune response against the tumor cells, and the potential of alphaviral vectors to eradicate tumor cells has been evaluated in several preclinical studies.

Chapter 3 describes a series of tumor treatment studies in mice performed with SFV-enhE6,7, determining the efficacy of subcutaneous (s.c.) administration of SFV-enhE6,7 in eradicating established HPV-dependent tumors. Previously, it was demonstrated that an HPV-specific immune response could be induced

upon administration of recombinant SFV expressing HPV16 E6 and E7 (SFV-E6E7) to mice.[12] Subsequently, a novel construct encoding a fusion protein of E6 and E7 under control of a translational enhancer was generated (SFV-enhE6,7), resulting in more potent CTL responses upon immunization in mice, compared to immunization with SFV expressing the separate proteins.[13]

Here, we demonstrate that all mice immunized s.c. with $5x10^6$ SFV-enhE6,7 two days after tumor inoculation and boosted on days 7 and 14 eliminated the tumor and remained tumor-free. When immunization was initiated at later time points (days 7-14-21 or 14-21-28 after tumor inoculation) 67% and 33% of the mice, respectively, remained tumor-free. That not all mice remained tumor-free in these latter two groups can presumably be ascribed to the very fast growth of the tumor. Most tumors initially regress, yet tumor growth appears to outpace the immune response at these time points.

Notably, all mice that eradicated the tumor remained tumor-free upon a second tumor challenge 3 months later, suggesting that HPV-specific memory CTLs were still present at this time point. In addition, CTL responses determined at very late time points after immunization and tumor challenge demonstrated that up to 340 days after immunization high levels of CTL activity could be determined. Thus, immunization with SFV-enhE6,7 results in a potent and long-term memory CTL response that correlates with a strong anti-tumor effect.

In **Chapter 4** it is demonstrated that the magnitude of the induced CTL activity and anti-tumor responses against HPV16-dependent tumors is not only determined by the immunization dose of SFV-enhE6,7 but also by the route of administration. We show that the i.v. and i.m. routes of injection are far more effective than the s.c. and i.p. routes of injection, both with respect to CTL induction and with respect to therapeutic anti-tumor responses. These differences in gene expression may influence the type and magnitude of the immune response. Despite the fact that the bulk CTL responses between the tested immunization routes do not differ significantly, tetramer analysis revealed that the i.v. and i.m. routes of immunization result in significantly higher pCTL frequencies. Indeed, a further analysis demonstrated that over 100-fold fewer virus particles are needed when immunizing i.v. or i.m. as opposed to the s.c. route.

These results are in agreement with observations of Colmenero *et al.* who demonstrated that the location of viral RNA differs after injecting rSFV via different injection routes. They showed that upon i.v. injection, rSFV-RNA distributes to a variety of different tissues, whereas it remains confined more locally after i.m. and s.c. injection. Upon i.v. and i.m. injection, but not upon s.c. injection, rSFV-RNA

can be detected in the spleen.[14]

Tumor treatment experiments show that i.v. and i.m. immunizations also result in superior anti-tumor responses compared with s.c. immunization, which can most likely be ascribed to the higher pCTL frequencies generated. Furthermore, the tumor treatment experiments clearly demonstrate the enormous potency of the vector. Exponentially growing tumors of approximately 500 mm^3 in size were seen to completely resolve and even some tumors as large as 1500 mm^3 decreased to one-third of their size. Another important observation is that even 22 weeks after immunization most of the mice are able to eradicate s.c. inoculated tumors, demonstrating a strong memory response induced upon immunization with SFV-enhE6,7.

In **Chapter 5**, we investigated whether the robust immune response elicited with SFV-enhE6,7 was strong enough to break immune tolerance in HPV16-E6/E7-transgenic mice. Here, it is shown that SFV-enhE6,7 did efficiently prime CTL activity in these mice, while others demonstrated that conventional DNA- or protein-based vaccines were unable to induce CTLs in these HPV16-E6/E7-transgenic mice.[15;16]

In contrast to wild-type mice, in these transgenic mice immunization via the s.c. route resulted in higher CTL responses than i.v. or i.m. immunizations. This could be explained by the following observations. These HPV16-E6/E7-transgenic mice express E6 and E7 under control of the K10 promoter in keratinocytes, leading to strong E6/E7-specific CTL tolerance.[15;17] Fausch *et al.* demonstrated that human Langerhans cells (LCs), which reside in the epidermis of the skin or in the epithelia of mucosal tissues, are not activated by uptake of HPV capsids, resulting in an inhibition of the HPV-specific immune response. Thus, LCs can be considered to be (co)responsible for the induction and maintenance of HPV tolerance.[18] By immunizing s.c. with SFV-enhE6,7, strong "danger signals" are provided that may turn immature, tolerizing LCs into mature, activating LCs.[7;19] In fact, Johnston *et al.* demonstrated that *in vivo* epidermal infection with SFV significantly increases the expression of MHC II, CD54 and CD80 on LCs, leading to maturation of the local LCs.[20] One could therefore speculate that s.c. immunization of the transgenic mice with SFV-enhE6,7 might result in maturation and activation of skin LCs and disruption of immune tolerance. Upon i.v. and i.m. injection, SFV-infected cells are not likely to reach LCs.

Although the mechanism and kinetics of tolerance in this mouse model certainly differ from that in the human clinical situation, these studies indicate that immunization with the SFV-enhE6,7 is extremely potent and therefore suggest that

the vector might also be effective in patients with HPV-induced cervical lesions.

Chapter 6 describes studies exploring the efficacy of SFV expressing IL12 to augment the antigen-specific immune and anti-tumor responses induced upon immunization with SFV-enhE6,7. Although immunization and boosting with SFV-enhE6,7 did prime CTL activity in HPV16-E6/E7-transgenic mice, the levels of specific cytolysis induced upon SFV-enhE6,7 were lower than those induced in wild-type mice. Therefore, we investigated whether co-expression of an immuno-stimulatory adjuvant along with SFV-mediated expression of E6,7 could further enhance the anti-tumor response. A promising adjuvant for cancer vaccination strategies is IL12.[21;22]

It is demonstrated that in wild-type mice the magnitude of this adjuvant effect of SFV-IL12 is dependent on its route of administration, the dose, and also on the timing of administration. In wild-type mice, SFV-enhE6,7-induced pCTL and CTL activity were enhanced by the addition of a low dose of SFV-IL12 to the s.c. prime immunization with SFV-enhE6,7. The observed enhancement in specific CTL activity was also reflected in the therapeutic effect. In transgenic mice, tolerant for HPV E6 and E7, the addition of SFV-IL12 to the priming SFV-enhE6,7 immunization also stimulated CTL responses. In conclusion, although our findings provide evidence for a specific enhancement of antigen-specific immune responses by SFV-IL12, prudence is called for when considering co-administration of SFV-IL12 to an immunization strategy, as enhancement of cell-mediated immune responses greatly depends on dosage and injection scheme.

In **Chapter 7** the efficacies of rSFV and a vector derived from adenovirus type 5 are compared for the induction of cellular and anti-tumor responses against HPV-induced cervical cancer. For this, a recombinant adenovirus was generated encoding the same HPV16 E6,7 construct as used in rSFV (Ad-enhE6,7). Here we show that immunization with SFV-enhE6,7 is more effective than immunization with Ad-enhE6,7. It resulted in two-fold higher pCTL frequencies and significantly higher levels of CTL activity. In addition, superior therapeutic effect was seen after SFV-enhE6,7 immunization at 100-1000-fold lower doses compared to Ad-enhE6,7 immunization.

To explore the possible mechanisms that might explain the difference in efficacy between both vectors, several aspects were studied. First, the role of CD4+ and CD8+ T lymphocytes in the anti-tumor response was investigated. Upon immunization with both rSFV and rAd, CD8+ T-cells appear to be the main effector cells involved in the protection against tumor growth. Next, the amount

of antigen expressed *in vivo* was determined using rSFV and rAd expressing luciferase. Intramuscular injection of $1x10^6$ SFV-luc and $5x10^8$ Ad-luc resulted in comparable levels of luciferase in the muscles of the mice. However, Sato *et al.* showed that transgene expression in cells infected with rAd may last for several weeks, whereas cells infected with rSFV die through apoptosis two to three days after infection.[23] Finally, the influence of possible anti-vector responses on booster injections was investigated. In contrast to the almost complete inhibition observed with rAd, pre-injection with rSFV resulted in a 1-2 log decrease. We hypothesize that following immunization with rAd the induction of CTL memory is disturbed by at least two mechanisms. First, strong anti-vector responses induced after immunization with rAd may prohibit the effect of boosting, necessary for good memory CTL induction[13], and secondly the prolonged expression of antigen with rAd may lead to the induction of effector T cells, which fail to acquire the key properties of memory cells.[24;25] In conclusion, the SFV vector system proved significantly more immunotherapeutic than the adenoviral vector system.

Chapter 8 describes a heterologous prime-boost strategy with SFV-enhE6,7 and E7-virosomes. This strategy results in higher numbers of antigen-specific pCTLs in mice than homologous immunization protocols. Most effective was a virosome prime followed by an rSFV boost. Yet, the induction of high numbers of pCTLs did not correlate with improved cytolytic responses. It did not result in CTLs with an enhanced responsiveness to *in vitro* antigenic stimulation, nor did it result in improved cytolytic activity or superior anti-tumor responses *in vivo* compared to an homologous prime-boost protocol with SFV-enhE6,7. However, the responses were higher than those induced by homologous prime-boosting with E7-virosomes.

Evasion of vector-specific immunity, induced by the prime immunization, is often considered the most important mechanism by which heterologous prime-boost immunization protocols induce superior immune responses.[26;27] To explore this issue for rSFV, irrelevant rSFV was mixed with E7-virosomes during the priming immunization followed by a SFV-enhE6,7 boost. Although this addition resulted in a reduced number of specific pCTLs, no difference in cytolytic activity of CTLs induced was found. These observations imply that rSFV is a very potent vector in homologous prime-boost strategies that does not need heterologous priming or boosting.

Chapter 9 presents a general discussion of the results described in the thesis. The preclinical studies described here show that SFV-enhE6,7 is able to elicite robust

therapeutic immune responses in mice, not hindered by anti-vector immunity. Also, SFV-enhE6,7 has the ability to turn an immune-compromised state toward immune-activation in immune-tolerant HPV-transgenic mice, underlining the exquisite potency of SFV-enhE6,7 for immunization purposes in patients with HPV-induced lesions. Finally, the SFV vector system proves significantly more immunotherapeutic than the adenoviral vector system.

Currently, two prophylactic HPV vaccines, Gardasil® and Cervarix®, are being implemented in many countries. Both vaccines induce full protection from cervical dysplasia associated with the two high-risk HPV types incorporated in the vaccine. Since these two high-risk HPV types are responsible for 70% of the cervical carcinomas approximately one third of the cervical cancers will still develop despite vaccination. Furthermore, these prophylactic vaccines are not effective in women already infected with HPV. Therapeutic HPV vaccines could fill in this niche of prophylactic vaccination, by attacking already established HPV infection and HPV-induced disease. In addition, these therapeutic vaccines could also represent a less invasive and disfiguring treatment option for women with pre-existing HPV-associated lesions, and may prevent recurrence or persistence of the disease.

It is difficult to predict how rSFV will perform in humans. The preclinical studies described in this thesis are very promising and fully justify clinical trials to evaluate the efficacy of rSFV to treat patients with (pre)malignant cervical lesions. A possible hurdle to clinical application of rSFV is that this alphavirus vector system has not yet been used in a clinical setting. This may slow down procedures involved in obtaining approval for initial clinical trials. However, if promising results emerge from early trials, possible reservations with respect to these vectors will likely diminish.

At the time an effective therapeutic vaccine can be launched, together with the prophylactic vaccines on a worldwide scale, this is expected to result in significant reduction of health-care costs and a further reduction of cervical cancer incidence and death.

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Nederlandse Samenvatting

Beknopt historisch overzicht van de strijd tegen baarmoederhalskanker

Baarmoederhalskanker is lange tijd één van de belangrijkste doodsoorzaken onder vrouwen geweest. Verscheidene ontwikkelingen hebben de afgelopen eeuw echter gezorgd voor een reductie van het aantal ziektegevallen, althans in de ontwikkelde landen.

In 1941 introduceert Georgious Papanicolaou een kleuringstechniek die premaligne en maligne epitheelcellen kan aantonen in uitstrijkjes van de baarmoederhals. De implementatie van deze zogenoemde "Pap-smears" markeert het begin van preventieve maatregelen tegen baarmoederhalskanker. Sindsdien is de incidentie van baarmoederhalskanker met 75% afgenomen in landen waar wordt gescreend met deze methode.

In 1991 ontdekt Zur Hausen dat er een verband bestaat tussen baarmoederhalskanker en het humane papillomavirus (HPV). Verder onderzoek wijst uit dat een persisterende infectie met zogenaamde hoogrisico typen van HPV noodzakelijk is voor het ontstaan van baarmoederhalskanker. De twee belangrijkste hoogrisico HPVs zijn type 16 en 18; samen veroorzaken zij ongeveer 70% van het totale aantal gevallen van baarmoederhalskanker.

Recentelijk zijn er moleculaire testen op de markt gekomen, waarmee HPV DNA in epitheelcellen van de baarmoederhals kan worden aangetoond. Deze techniek is gevoeliger dan het Pap-uitstrijkje. Nadeel is wel dat de testen alleen het virale DNA aantonen en niet de maligne transformatie. Hoe het Pap-uitstrijkje het best met HPV DNA-testen kan worden gecombineerd voor screening, is op dit moment onderwerp van een uitgebreid onderzoek.

Ondanks al deze ontwikkelingen blijft baarmoederhalskanker de op één na meest voorkomende vorm van kanker onder vrouwen wereldwijd. Ongeveer de helft van de vrouwen met baarmoederhalskanker overlijdt hier ook aan. Meer dan 80% van alle gevallen wordt gevonden in de ontwikkelingslanden, waar men niet beschikt over goede preventieve zorg.

Maar ook in landen waar uitstekende screeningsmogelijkheden zijn worden nog steeds aanzienlijke aantallen vrouwen gediagnostiseerd met baarmoederhalskanker. Een belangrijke oorzaak hiervoor is non-compliance, vaak veroorzaakt door angst of gêne bij een vaginaal onderzoek. Daarom blijft het nodig dat er onderzoek wordt verricht om de preventie en behandeling van baarmoederhalskanker waar mogelijk te verbeteren.

Zoals vermeld is een persisterende infectie met hoogrisico HPV essentieel voor het ontstaan van baarmoederhalskanker. Daarom is vaccinatie waarschijnlijk de meest effectieve manier om infectie met HPV en daarmee de door HPV veroorzaakte

ziekte te voorkomen. Op dit moment zijn er twee profylactische vaccins tegen baarmoederhalskanker op de markt: Gardasil® en Cervarix®. Deze vaccins bieden bescherming tegen de hoogrisico HPV typen 16 en 18.

De huidige profylactische vaccins bieden alleen bescherming tegen de twee typen HPV die in het vaccin zijn opgenomen. Dit betekent dat, ondanks vaccinatie, nog steeds 30% van de gevallen van baarmoederhalskanker zal blijven ontstaan. Verder hebben deze profylactische vaccins geen therapeutische werking, met als gevolg dat alle vrouwen die al zijn geïnfecteerd met HPV er geen baat meer bij hebben. Een therapeutisch vaccin tegen baarmoederhalskanker en de voorstadia van deze ziekte zou een toevoeging van betekenis kunnen zijn in de strijd tegen baarmoederhalskanker.

Introductie

Het doel van deze studie, beschreven in dit proefschrift, was het vaststellen van de effectiviteit van een therapeutische vaccinatiestrategie tegen baarmoederhalskanker en voorstadia van deze ziekte (zogenaamde CIN laesies). Baarmoederhalskanker wordt veroorzaakt door een persisterende infectie met een hoogrisico HPV. Twee van de vroege oncogene eiwitten van deze hoogrisico HPV-typen, E6 en E7, kunnen een interactie aangaan met respectievelijk de cellulaire eiwitten p53 en pRb. Deze laatstgenoemde eiwitten zijn medeverantwoordelijk voor een goede regulatie van de cyclus van de gastheercel. De interactie tussen E7 en pRb resulteert in een verhoogde DNA replicatie en de interactie tussen E6 en p53 voorkomt apoptose (celdood) van de cel. Dit kan leiden tot een maligne transformatie van deze geïnfecteerde cellen. Een aanhoudende productie van deze oncogene eiwitten E6 en E7 is een vereiste voor het behoud van de maligne transformatie en daarmee de ontwikkeling van baarmoederhalskanker. Aangezien E6 en E7 op alle kankercellen tot expressie worden gebracht zijn deze eiwitten aantrekkelijke aangrijpingspunten voor immunotherapeutische strategieën.

De in dit proefschrift beschreven therapeutische vaccinatiestrategie is gebaseerd op de toepassing van een recombinante virale vector, het zogenaamde Semliki Forest virus (SFV). De gebruikte SFV-vector brengt een fusie-eiwit van E6 en E7 tot expressie (SFV-enhE6,7). De doeltreffendheid van dit therapeutische vaccin werd in een aantal preklinische studies in een muizenmodel onderzocht.

Hoofdstuk 1 geeft een algemene introductie over baarmoederhalskanker (cervixcarcinoom), de etiologische rol van HPV infectie in het ontstaan van (pre) maligne laesies, en de huidige behandelingsmogelijkheden tegen de verschillende stadia van de ziekte. Vervolgens wordt de rol van het immuunsysteem bij de

ontwikkeling van de ziekte beschreven. Tenslotte volgt een overzicht van de huidige beschikbare profylactische en therapeutische vaccinatiemogelijkheden, zowel de geregistreerde als de nog experimentele.

Wereldwijd is baarmoederhalskanker de op één na belangrijkste vorm van kanker onder vrouwen. Door de integratie van de virale vroege eiwitten E6 en E7 in het genoom van de gastheercel, zijn hoogrisico HPV, met name typen 16, 18, 31, 33 en 45 in staat tot maligne transformatie van de epitheelcellen van de baarmoederhals. Om het getransformeerde fenotype van de cellen te behouden is continue expressie van E6 en E7 noodzakelijk. Daarom kunnen E6 en E7 worden gezien als tumorspecifieke antigenen. Dit maakt E6 en E7 zeer geschikte targets voor immunotherapeutische behandeling van baarmoederhalskanker.

Therapeutische immunisatie tegen virus-geïnfecteerde cellen of tumorcellen beoogt de inductie van cytotoxische T-lymfocyten (CTL). Deze CTLs kunnen specifiek de geïnfecteerde cellen of tumorcellen herkennen en lyseren. T-helpercellen en geactiveerde antigeen-presenterende cellen (APCs), in het bijzonder dendritische cellen, zijn essentieel voor het aanzetten tot differentatie en de expansie van antigeen-specifieke CTLs, en voor de vorming van geheugencellen ("memory CTLs").

Het merendeel van de infecties met HPV zijn van voorbijgaande aard en geven geen aanleiding tot de vorming van detecteerbare cervicale laesies. Echter, HPVs zijn zodanig geëvolueerd dat ze verschillende mechanismen bezitten, waarmee ze het immuunsysteem kunnen omzeilen. Hierdoor worden geïnfecteerde cellen en (pre-)maligne cellen niet altijd herkend door het immuunsysteem. Verder wordt aangenomen dat patiënten met CIN-laesies of baarmoederhalskanker immuungecompromitteerd zijn en/of een bepaalde immunologische tolerantie hebben ontwikkeld tegen HPV. Voor een succesvolle therapeutische vaccinatiestrategie betekent dit, dat er een zeer potent vaccin nodig is, zodat de balans kan omslaan van immunologische tolerantie naar activatie.

Hoofdstuk 2 biedt een overzicht van recombinante alfavirale vectoren die worden gebruikt in antitumor en antimicrobiële immunotherapie. Alfavirussen zijn kleine positieve RNA membraanvirussen, die behoren tot de familie van de *Togaviridae*. De meest gebruikte vectoren zijn afgeleid van het Semliki Forest virus (SFV), het Venezuelaanse paardenencefalitis virus (VEE) en het Sindbis virus (SIN). Met deze vectoren kunnen recombinante virusdeeltjes worden gemaakt die in staat zijn om één ronde van infectie te geven. Deze vectoren worden replicatiedefectief genoemd, omdat na deze ene ronde van infectie er geen nieuwe virusdeeltjes gevormd kunnen worden. Het recombinante RNA van de vectoren is zelfreplicerend

en de vreemde genen worden maar tijdelijk tot hoge expressie gebracht.

Alfavirale vectoren staan sterk in de belangstelling, omdat ze in staat zijn een hoge humorale en cellulaire immuunrespons op te wekken. De karakteristieke eigenschappen die alfavirussen tot aantrekkelijke kandidaten maken voor het gebruik bij mensen zijn; (i) recombinante alfavirussen geven hoge expressie van de gecodeerde eiwitten, (ii) na 48-72 uur sterven de geïnfecteerde cellen door apoptose (de apoptotische cellen bevatten grote hoeveelheden van het transgene eiwit wat gunstig is voor het opwekken van een immuunrespons via "cross-priming"), (iii) recombinante alfavirussen kunnen zowel het aangeboren ("innate") als het verworven ("acquired") immuunsysteem activeren.

Verscheidene immunisatiestrategieën tegen infectieziekten hebben laten zien dat de humorale immuunrespons, opgewekt door alfavirussen, sterk varieert en daarnaast afhankelijk is van het type antigeen, de verwerking/presentatie van het antigeen en de toedieningsweg. Desalniettemin wordt in de meeste studies aangetoond dat alfavirussen sterke cellulaire immuunresponsen induceren die kunnen beschermen tegen specifieke micro-organismen of tumoren. De doeltreffendheid van recombinant SFV is in verschillende preklinische studies geëvalueerd.

Hoofdstuk 3 beschrijft een serie experimenten waarin gekeken werd naar het effect van de behandeling met SFV-enhE6,7 van tumoren in muizen. In dit model worden HPV-getransformeerde tumorcellen subcutaan (s.c.) geïnjecteerd bij muizen, die vervolgens uitgroeien tot een tumor. Tumor-dragende muizen werden geïmmuniseerd met SFV-enhE6,7 en vervolgens werd gekeken of door deze immunisatie de tumorgroei wordt geremd. Voorafgaand aan dit onderzoek was reeds aangetoond dat met behulp van SFV, coderend voor E6 en E7, een sterke HPV-specifieke immuunrespons kan worden geïnduceerd in muizen.

In dit hoofdstuk wordt aangetoond dat muizen, die s.c. geïmmuniseerd zijn met SFV-enhE6,7 in staat zijn tumorcellen op te ruimen en daarna tumorvrij te blijven. Belangrijk is dat deze tumorvrije muizen ook tumorvrij blijven na een tweede injectie met tumorcellen drie maanden later. Dit suggereert dat er op dit late tijdstip nog steeds HPV-specifieke geheugen-CTLs aanwezig zijn. Verder blijkt uit het onderzoek dat zelfs 340 dagen na immunisatie nog sterke HPV-specifieke CTL-responsen aantoonbaar zijn. Samenvattend kan gesteld worden dat immunisatie met SFV-enhE6,7 leidt tot een potente CTL-respons, die gepaard gaat met een sterk antitumor effect. Daarnaast geeft deze immunisatie ook een langdurige geheugen CTL-respons.

Uit experimenten beschreven in **hoofdstuk 4** blijkt dat de sterkte van de, door SFV-enhE6,7, opgewekte immuun- en antitumorrespons tegen HPV-geïnduceerde tumoren, niet alleen dosisafhankelijk is, maar ook afhangt van de toedieningsroute van het vaccin.

In deze studie werd vastgesteld dat i.v. en i.m. toediening van SFV-enhE6,7 vele malen effectiever is dan s.c. of intraperitoneale (i.p) toediening. Dit geldt zowel voor CTL inductie als therapeutische antitumorresponsen. Klaarblijkelijk beïnvloedt de plaats van genexpressie de activatie van de immuunrespons. Hoewel er geen significant verschil te meten was in de CTL activiteit, liet analyse met behulp van MHC-tetrameren zien dat i.v. en i.m. toediening een significant hogere precursor CTL (pCTL) frequentie oplevert. Verdere analyse liet zien dat, om dezelfde CTL activiteit te induceren, ruim honderd keer minder virusdeeltjes nodig zijn bij i.v. of i.m. toediening in vergelijking met s.c. of i.p. toediening.

Tumorbehandelingsexperimenten lieten zien dat i.v. en i.m. immunisaties ook een betere antitumorrespons geven, vergeleken met s.c. immunisatie zoals in hoofdstuk 3 beschreven. Dit kan hoogstwaarschijnlijk worden toegeschreven aan de hogere pCTL frequenties die worden gegenereerd. Verder wordt met deze tumor-experimenten de enorme potentie van de SFV vector aangetoond. Exponentieel groeiende tumoren, tot zo'n 500 mm^3 in grootte, kunnen volledig in remissie gaan en zelfs tumoren van 1500 mm^3 kunnen soms tot een derde van hun grootte worden terug gebracht. Een andere belangrijke observatie is dat zelfs 22 weken na de laatste immunisatie de meeste muizen nog steeds in staat zijn om de geïnjecteerde tumoren op te ruimen.

In **hoofdstuk 5** werd onderzocht of de immuunrespons die door SFV-enhE6,7 wordt opgewekt sterk genoeg is om de immunologische tolerantie in HPV16-E6/E7-transgene muizen te doorbreken. Immunisatie met SFV-enhE6,7 blijkt inderdaad een specifieke CTL-respons te induceren in deze transgene muizen, terwijl andere immunisatiestrategieën gebaseerd op conventionele DNA- of eiwitvaccins dit niet kunnen bewerkstelligen in deze transgene muizen.

In tegenstelling tot wat we zien bij wildtype muizen, geeft niet i.m. of i.v. toediening maar s.c. immunisatie bij HPV-transgene muizen de beste CTL-respons. De volgende bevindingen kunnen dit mogelijk verklaren. Eerder is beschreven dat HPV16-E6/E7-transgene muizen E6 en E7 tot expressie brengen op het celoppervlak van keratinocyten, wat leidt tot sterke perifere tolerantie van de E7-specifieke CTLs. Fausch *et al.* hebben aangetoond dat humane Langerhans cellen, die gelokaliseerd zijn in de epidermis van de huid of in het epitheel van de slijmvliezen, niet worden geactiveerd door de opname van de capside-eiwitten van

HPV. Dit heeft tot gevolg dat de HPV-specifieke immuunrespons wordt geremd. Langerhans cellen zouden dus mede verantwoordelijk kunnen zijn voor de inductie en het behoud van de tolerantie tegen HPV. Bij s.c. immunisatie met SFV-enhE6,7 worden "danger-signals" afgegeven die onrijpe Langerhans cellen, die tolerantie veroorzaken, omzetten naar rijpe activerende Langerhans cellen. Deze hypothese wordt ondersteund door bevindingen van Johnston *et al.* Deze auteurs laten zien dat SFV infectie van de epidermis *in vivo* een significant verhoogde expressie van MHC II, CD54 en CD80 op de Langerhans cellen geeft. Hieruit valt af te leiden dat s.c. immunisatie met SFV-enhE6,7 de Langerhans cellen in de huid van transgene muizen kan aanzetten tot rijping en activatie. Op die manier verstoren deze cellen de immunologische tolerantie. Via i.v. of i.m. toediening is het niet waarschijnlijk dat deze Langerhans cellen worden bereikt.

Het mechanisme van tolerantie in transgene muizen verschilt waarschijnlijk wezenlijk van de tolerantie die kan optreden na een humane HPV-infectie bij de mens. De hoop en de verwachting is dat ook de lichte mate van immunotolerantie bij patiënten met HPV-geïnduceerde laesies doorbroken kan worden door immunisatie met SFV-enhE6,7.

In **hoofdstuk 6** werd onderzocht of toevoeging van een tweede SFV vector, die interleukine 12 (IL12) tot expressie brengt, de SFV-enhE6,7-geinduceerde antigeen-specifieke immuun- en antitumorrespons verder kan versterken. In het vorige hoofdstuk werd aangetoond dat het mogelijk is om in HPV16-E6/E7-transgene muizen met een immunisatie met SFV-enhE6,7 een E7-specifieke CTL-respons te induceren. Deze respons blijft echter lager dan die in wildtype muizen. In dit hoofdstuk werd onderzocht of door toevoeging van een immuunstimulerend adjuvant de geïnduceerde immuun- en antitumorrespons kan worden vergroot.

In wildtype muizen is het adjuvante effect van SFV-IL12 afhankelijk van zowel de dosis als de timing van toediening. Een lage dosering van SFV-IL12, toegevoegd aan de eerste SFV-enhE6,7 immunisatie, geeft aanleiding tot een verhoogde antigeen-specifieke CTL-respons. Het positieve effect op de specifieke CTL activiteit bleek overeen te komen met een verhoogd therapeutisch effect. Verder werd ook in HPV-transgene muizen een verhoging van de antigeen-specifieke CTL-respons gezien, na toevoeging van SFV-IL12 aan de eerste immunisatie met SFV-enhE6,7.

Hoewel SFV-IL12 de antigeen-specifieke immuunrespons enigszins versterkt lijkt, op basis van deze experimenten, toevoeging van SFV-IL12 aan immunisatieprotocollen niet gerechtvaardigd. Het risico bestaat dat de immuunrespons wordt geremd in plaats van verhoogd.

in vivo wordt er geen verhoogde cytolytische activiteit of betere antitumorrespons gezien vergeleken met een homologe immunisatiestrategie met SFV-enhE6,7. Daarentegen zijn de resultaten wel beter dan die na een homologe prime-boost immunisatie met E7-virosomen.

Met heterologe prime-boost immunisatiestrategieën kan de immuunrespons tegen de vector worden omzeild. Dit wordt gezien als het meest belangrijke mechanisme op basis waarvan met deze immunisatiestrategieën sterkere immuunresponsen kunnen worden opgewekt. In deze studie is ook gekeken of deze vector-specifieke immuniteit een belangrijke rol speelt bij immunisatiestrategieën met rSFV. Om dit effect te onderzoeken werd aan de immunisatie met E7-virosomen irrelevant rSFV toegevoegd. Na deze immunisatie volgde een booster-immunisatie met SFV-enhE6,7. De toevoeging van irrelevant rSFV aan de immunisatie gaf verminderde aantallen specifieke pCTLs. Er kon echter geen verschil worden aangetoond in de cytolytische activiteit van de geïnduceerde CTLs vergeleken het oorspronkelijke heterologe prime-boost protocol: E7 virosomen gevolgd door SFV-enE6,7. Deze studie laat zien dat een immuunrespons tegen de SFV-vector de therapeutische werking niet hindert waardoor SFV-enhE6,7 uitstekend in homologe immunisatieprotocollen toe te passen is.

Hoofdstuk 9 presenteert een algemene discussie van de resultaten in de verschillende hoofdstukken van dit proefschrift. Hiernaast worden de mogelijkheden en uitdagingen van een eventuele introductie van een therapeutisch HPV vaccin in perspectief gebracht, in het bijzonder die van een therapeutisch vaccin gebaseerd op het gebruik van het recombinante SFV-vectorsysteem. Het geheel wordt belicht tegen de achtergrond van de huidige implementatie van de profylactische HPV vaccinatie in veel landen.

De krachtige therapeutische immuunresponsen die kunnen worden opgewekt met SFV-enhE6,7, de afwezigheid van pre-existerende antilichamen tegen SFV onder mensen, de mogelijkheid om rSFV te gebruiken in homologe immunisatieprotocollen en de veiligheid van het SFV-vectorsysteem, maken rSFV tot een serieuze kandidaat voor klinische evaluatie als mogelijke behandelingvorm voor (pre)maligne CIN laesies en vroeg-invasieve baarmoederhalskanker.

De samenvatting begon met een overzicht van de mogelijke maatregelen tegen baarmoederhalskanker die zijn geïmplementeerd in de afgelopen decennia. Deze maatregelen zijn zeer succesvol gebleken en hebben de incidentie van baarmoederhalskanker substantieel verminderd. Dit geldt echter alleen voor de ontwikkelde landen. Meer dan 80% van de gevallen van baarmoederhalskanker komen echter voor in ontwikkelingslanden. De verwachting is dat dit percentage zal

In **hoofdstuk 7** wordt de cellulaire en antitumorrespons na immunisatie met rSFV vergeleken met de respons opgewekt door immunisatie met een adenovirus-vector. Hiertoe werd een recombinant adenovirus type 5 geconstrueerd (Ad-enhE6,7) met hetzelfde HPV16-E6,7 construct als SFV-enhE6,7. Immunisatie met SFV-enhE6,7 resulteert in een twee keer hogere pCTL frequentie en een significant hogere CTL activiteit vergeleken met immunisatie met Ad-enhE6,7. Ook is het therapeutisch effect van immunisaties met SFV-enhE6,7 significant beter.

Er zijn verschillende mogelijke mechanismen, die aan dit verschil tussen rSFV en rAd ten grondslag kunnen liggen. Als eerste werd onderzocht welke rol CD4+ en CD8+ T-cellen spelen in de antitumorrespons. Na immunisatie met zowel rSFV als rAd, spelen de CD8+ T-cellen de belangrijkste rol in de bescherming tegen tumoruitgroei. Ten tweede is gekeken naar de hoeveelheid antigeen die *in vivo* tot expressie komt na immunisatie met rSFV en rAd. I.m. toediening van $1x10^6$ i.u. rSFV en $5x10^8$ i.u. rAd geven aanleiding tot vergelijkbare hoeveelheden antigeenexpressie in de spieren van de muizen. Sato *et al.* laten in hun onderzoek zien dat de antigeenexpressie in rAd-geïnfecteerde cellen wekenlang kan voortduren. Dit in tegenstelling tot de expressie in cellen geïnfecteerd met rSFV, die na twee à drie dagen in apoptose gaan. Als laatste is gekeken naar de invloed van een mogelijke antivectorrespons, die opgewekt zou kunnen worden bij de eerste immunisatie. In tegenstelling tot een bijna volledige remming van antigeenexpressie door een tweede dosis rAd resulteert een pre-injectie van rSFV slechts in een partiële verlaging van de antigeenexpressie.

Op basis van deze studie veronderstellen we dat immunisatie met rAd niet resulteert in de inductie van CTL geheugencellen. De sterke antivectorrespons, die wordt opgewekt door de eerste immunisatie met rAd verhindert een effectieve booster-immunisatie. Een booster-immunisatie is noodzakelijk voor een goede geheugenrespons. Daarnaast kan de langdurige expressie van antigeen, na immunisatie met rAd, resulteren in de inductie van effector-T-cellen die tijdelijk actief zijn maar zich niet kunnen ontwikkelen tot functionele geheugencellen.

In **Hoofdstuk 8** wordt een heterologe prime-boost strategie met SFV-enhE6,7 en E7-virosomen geëvalueerd. Uit andere studies blijkt dat heterologe prime-boost strategieën hogere aantallen antigeen-specifieke pCTLs induceren dan homologe immunisatieprotocollen. De studie beschreven in dit hoofdstuk laat zien dat een heterologe prime-boost immunisatiestrategie, waarin muizen eerst worden geïmmuniseerd met E7 virosomen en daarna worden geboosterd met SFV-enhE6,7, de hoogste aantallen pCTLs induceert. Deze hoge aantallen pCTLs leiden echter niet tot CTLs die sterker reageren op antigeenstimulatie. *In vitro* en

zijn toegenomen tot 90% in het jaar 2020. De vrouwen uit deze ontwikkelingslanden, die de meeste baat zouden hebben bij vaccinatie, zullen hoogstwaarschijnlijk geen toegang hebben tot zo'n vaccin omdat ze het zich niet kunnen veroorloven. Ook hebben zij geen toegang tot de huidige screeningsmogelijkheden, zoals de Pap-uitstrijkjes, vanwege gebrek aan geld of faciliteiten. Het vinden van mogelijkheden om ook meisjes en vrouwen uit deze achtergestelde landen toegang te bieden tot profylactische en, eventueel therapeutische vaccins is misschien nog wel de grootste uitdaging in de strijd tegen baarmoederhalskanker wereldwijd.

Dankwoord

In essentie geef je met danken aan dat je iets niet had kunnen bereiken zonder hulp. Over het algemeen kun je stellen dat je niets zonder betrokkenheid van een ander kunt bereiken. Had iemand mij 7 jaar geleden gezegd dat ik nu zou promoveren, al is het met hulp van zovele anderen, dan had ik diegene vol ongeloof aangekeken en misschien zelfs wel uitgelachen. Mijn gedachten gaan daarom, toch vooral terug naar mijn allereerste bezoeken aan het UMCG. Iemand heeft kennelijk toch iets gezien wat ik zelf toen nog niet zag. Het eerste jaar had ik nogal eens moeite om in te zien waar het allemaal tot zou lijden*. Al snel kreeg ik plezier in wat ik deed op het lab en voelde ik me meer thuis in deze wonderlijke wetenschappelijke wereld. Gedurende de jaren heb ik heel wat afgevraagd, uitgeplozen, besproken en nagelezen. Mijn verbale incontinentie heeft soms voor veel plezier en vast ook voor ergernis gezorgd. Bovenal ben ik dankbaar dat mijn eigen enthousiasme en nieuwsgierigheid me de drive hebben gegeven om telkens door te zetten. Dat ik nu dit kan schrijven, heb ik voor het grootste deel te danken aan de mogelijkheden die me zijn geboden, de hulp en steun (in de breedste zin van het woord) die ik heb mogen ontvangen en het vertrouwen van veel mensen. In de opsomming die gaat volgen zal ik niet iedereen die een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift bij naam kunnen noemen, maar ik ben hen allemaal erg dankbaar hiervoor.

Voorts wil ik met name bedanken;
Jan Wilschut, als zijnde mijn eerste promotor. Jouw enthousiasme en vertrouwen maakten dat ik niet lang na hoefde te denken toen je me een onderzoeksplaats aanbood tijdens mijn sollicitatie naar de opleiding tot arts-microbioloog. Dit was spannend voor ons allebei, gezien ik de eerste “arts” was die zich voegde bij jullie groep. Je begeleiding heb ik als zeer leerzaam ervaren, bedankt voor alles.

Toos Daemen, mijn dagelijkse begeleider en co-promotor. Jouw geduld en hulp hebben ervoor gezorgd dat ik me de, voor mij vreemde, taal waarin jullie spreken enigszins eigen heb kunnen maken. Je deur stond altijd open voor mijn vragen en je bent bij elke stap van mijn promotietraject zeer nauw betrokken geweest. Je prikkelende vragen en uitgesproken ideeën zijn essentieel geweest voor de vorming van dit proefschrift.

John Degener, mijn opleider en tweede promotor. Dank je voor alle ruimte en mogelijkheden die je hebt geschapen, om de uitdagende combinatie van de kliniek en de research tot een succes te maken.

Ate van der Zee, mijn derde promotor. Hartelijk dank voor jouw bijdrage aan dit proefschrift. Jouw praktische benadering vanuit de kant van de kliniek zorgt ervoor dat je niet uit het oog verliest voor wie je het doet, de patiënt.

Joke Regts, mijn steun en toeverlaat op de werkvloer. Van jou heb ik onder andere geleerd dat het begrip steriel werken verschillende betekenissen heeft. Ik heb bewondering voor je enorme nauwgezetheid, een kunst die leidt tot geweldig reproduceerbare resultaten. De samenwerking met jou heb ik als een bijzondere en dierbare tijd ervaren. Daarnaast ben ik je dankbaar dat je ook op deze belangrijke dag aan mijn zijde wilt staan.

Barry-Lee, Jørgen en Arjan, mijn kamergenoten. Ik kijk met veel plezier terug op de periode waarin we lief en leed met elkaar deelden. Inmiddels heb ik al jullie promoties bij mogen wonen en heb ik zo de kunst mooi kunnen afkijken.

Jolanda, Laura en Jeroen behoorden tot de "oude" AIO's groep. Jullie relativerings vermogen en de al opgebouwde ervaring gaven me op gezette tijden de rust en vertrouwen dat alles erbij hoort en het uiteindelijk weer op zijn pootjes terecht komt.

Natuurlijk waren er nog veel meer collega's bij de Moleculaire Virologie die ik hier niet wil overslaan. Annechien, Anke, Baukje-Nynke, Chekwa, Felix, Heidi, Hilde, Ingrid, Iza, Jacqueline, Judith, Marijke, Mateusz, Paul, Tjarko, Toon, Wouter. Jullie expertise heeft mij vaak verder geholpen en ik kijk met veel plezier terug naar de fijne periode die ik bij jullie heb gehad.

Bert Dontje, als de vaste biotechnicus. Niet alleen de muizen maar ook persoonlijk ben ik heel dankbaar voor jouw betrouwbare hulp en vaste hand. Daarnaast dank ik de medewerkers van het CDL voor de goede verzorging van al "mijn" muisjes. En natuurlijk dank ik alle muisjes, die niet geheel uit eigen wil, een essentiële bijdrage hebben geleverd aan dit proefschrift.

Hidde Haisma, Gera Kamps, Marianne Rots, en Ou Jianghua van de afdeling Therapeutisch Gen Modulatie. Heel erg bedankt voor alle hulp, zowel praktisch als in theorie, bij de experimenten met de adenovirale vectoren.

De medewerkers van de afdeling medische biologie, in het bijzonder Marja Brinker, wil ik bedanken voor de hulp bij het opzetten van de PCR voor het identificeren van de transgene muizen.

Geert Messander, bedankt voor al je ondersteunende hulp bij de FACS experimenten. Jan Wijbenga bedankt voor de goede zorgen over alle andere apparaten. En de afdeling ICT, in het bijzonder Guus van den Brekel, voor alle hulp als de computer weer iets anders in gedachten had dan ik.

Margaret Chen, thank you for contributing the SFV-IL12 virus particles and all the constructive comments on the paper. Prof. L. Gissmann and Dr. N. Michel for kindly supplying us with founder HPV-transgenic mice. Marc Fiedler and Pidder Jansen Dürr for the E7 protein you kindly provided.

Willem Manson, Jan Arends, Rik Winter, Nico Meessen en Greetje Kampinga, de

artsen-microbioloog van het UMCG. Jullie hebben allemaal zo je eigen specifieke kwaliteiten waar ik al behoorlijk veel van heb opgestoken. In de afgelopen 7 jaar, hebben jullie mij zien ontwikkelen van een ietwat naïeve "AIOS" naar een toekomstig collega, die niet bang is om voor haar mening op te komen.

Karola Waar, Niek Arents, Jutte de Vries, Glen Mithoe, Astrid Buss, Jerome Lo Ten Foe en Felix Geeraerdts, mijn collega arts-assistenten. Jullie hebben me voorzien van de nodige adviezen en mijn geweeklaag nu lang genoeg aangehoord. Ik stel het zeer op prijs dat jullie je, vooral in die hectische laatste maanden van de afronding, flexibel hebben opgesteld.

Verder wil ik alle andere collega's van de afdeling Medische Microbiologie in het UMCG bedanken. Ik kijk uit naar de nieuwe uitdagingen die in het verschiet liggen en heb er vertrouwen in dat we ook hier een succes van kunnen maken.

De collega's van het LVI wil ik bedanken voor de morele steun tijdens mijn stage. Voornamelijk, René Benne voor het beschikbaar stellen van de nodige tijd en begrip.

Ook ben ik de leden van de leescommissie Prof. H.J. Haisma, Prof. L.F.M.H. de Leij en Prof. P.J.F. Snijders zeer erkentelijk voor het beoordelen van het proefschrift en hun goedkeuring.

Wil en Thomas Lof, dankzij jullie heeft mijn proefschrift een persoonlijke omslag. Ook wil ik jullie bedanken voor het gebruik van het heerlijke pied-à-terre in Frankrijk en de relativerende grappen die vooral de laatste loodjes af en toe wat lichter maakten.

Peter van der Sijde, heel wat zorgen en stress zijn me bespaard gebleven door jouw expertise om het proefschrift mooi vorm te geven, bedankt hiervoor.

Marieke, Elbrig, Phillis en Paulien, mijn vriendinnen uit mijn tijd van de Geneeskunde studie in het AMC. Wie had gedacht dat ik als eerste zou promoveren (ik niet!). We zijn aardig verspreid geraakt over heel Nederland, desondanks hoop ik dat we nog lang een goed contact zullen houden.

Vera en Annelies, getuigen van alle belangrijke life-events tot nu toe, mijn vriendschap met jullie is me heel dierbaar. Vooral de laatste tijd heb ik jullie wel tekort gedaan. Maar jullie steevaste vertrouwen in mijn kunnen hebben er mede voor gezorgd dat ik heb doorgezet en dat we nu een mooi feest te vieren hebben.

Suzan, al vriendinnen vanaf ons 3e levensjaar. Ik vind het erg fijn dat onze vriendschap hier in Groningen is opgebloeid, we wonen weer net als vroeger op fietsafstand van elkaar. Naast David denk ik dat jij één van de weinige bent die me soms precies begrijpt zonder dat ik het hoef uit te leggen.

Een goede buur is beter dan een verre vriend. Dit geldt zeker voor een aantal

buren uit de Leeuwarderstraat. Ik heb genoten van de gezelligheid en heerlijke etentjes, die zorgen voor de broodnodige ontspanning en afleiding.

Guus en Joan, hartelijk dank voor het beschikbaar stellen van het heerlijk ontspannen toevluchtsoord in Denemarken. Guus bedankt voor het proeflezen van delen uit dit proefschrift. Hiernaast bewonder ik jullie enorme positieve levensinstelling. We komen zeker een keer langs in Afrika!

Mijn schoonfamilie heeft mij ook enorm geholpen. Jullie stonden altijd klaar voor mij en mijn gezin. De warme en hechte familieband heb ik leren waarderen en hoop ik mezelf eigen te kunnen maken. Ik ben jullie enorm dankbaar voor de ontelbare keren dat jullie ons te hulp schoten. Was het niet bij de verbouwing van het huis dan wel was het wel bij de opvang van de kinderen.

Mijn ouders ben ik dankbaar voor het bijbrengen van zelfdiscipline en doorzettingsvermogen. Jullie normen en waarden die ik heb meegekregen leggen hoge eisen op, maar ik heb geleerd dat je er zeer ver mee kunt komen. Daarnaast hoop ik nog lang te kunnen genieten van jullie als ouders en in de rol als opa en oma voor mijn beide dochters. Jan Derk, mijn broer, het spijt me dat ik het je als oudere zus weer lastig maak door te promoveren, maar ik heb alle vertrouwen in je. Ik vind het heel fijn, dat je aan mijn zijde wilt staan op deze dag, dank je. Verder, ben ik mijn hele familie dankbaar voor de steun en aanwezigheid in mijn leven.

Mijn bewondering gaat uit naar al die mensen die dagelijks op zoek zijn naar de waarheid in wetenschappelijke vraagstukken. Door hun ideeën, onderzoeken en artikelen komen we telkens weer een stapje dichterbij geweldige vernieuwingen. Die door de juiste vertaalslag, een beetje geluk en een flink portie idealisme, voor een enorm verschil kunnen zorgen in het leven van zo vele mensen.

Dankbaar ben ik uiteraard voor mijn gezin. David, Ruth, Hannah en Dorus, wil ik bedanken voor de onvoorwaardelijke liefde die ik van jullie elke dag weer mag ontvangen. Het geeft mij de kracht om door te zetten en te gaan voor wat ik denk dat belangrijk is en daarmee hopelijk een beetje verschil uitmaak. Tot slot, David, mijn nummer 1, samen met jou voel ik me compleet en durf ik alles aan. Ik hoop nog heel lang samen met jou en onze kinderen van het leven te kunnen genieten.

Annelies Riezebos-Brilman werd geboren op 3 juni 1975, te Nijmegen en groeide op in Oldenzaal. In 1993 behaalde zij haar VWO examen aan het Twents Carmel Lyceum te Oldenzaal.

Na haar middelbare school vertrok zij naar Amsterdam en startte hier met de studie Medische Biologie aan de Universiteit van Amsterdam. In 1994 sloot zij het eerste studiejaar af met de propedeuse Medische Biologie en werd zij tevens ingeloot voor de studie Geneeskunde in het AMC. Hier behaalde zij haar doctoraal examen in 1998, gevolgd door het artsexamen in 2001.

Na haar artsexamen verhuisde ze naar Groningen. In het UMCG werd op 1 september 2001 begonnen met de opleiding tot arts-microbioloog onder supervisie van Prof. Dr. J.E. Degener. De opleiding startte met 9 maanden onderzoek bij de sectie Moleculaire Virologie. Na deze 9 maanden werd de opleiding uitgebreid met twee jaar om verder te werken aan het onderzoek naar een therapeutische vaccinatiestrategie tegen baarmoederhalskanker. Het wetenschappelijk onderzoek resulteerde in dit proefschrift onder leiding van Prof. Dr. J.C. Wilschut, Prof. Dr. A.G.J. van der Zee, Prof. Dr. J.C. Degener en Dr. C.A.H.H. Daemen. Als lid van de onderwijscommissie van de NVAMM is zij actief betrokken geweest bij het opstellen van het huidige logboek voor de opleiding tot arts-microbioloog, vanaf de oprichting van deze commissie in 2002 tot 2006. Van november 2005 tot en met oktober 2006 was zij gedetacheerd op het Laboratorium voor Infectieziekten in Groningen voor het deel klinische virologie van haar opleiding. Momenteel is zij in het UMCG bezig met het laatste jaar van haar opleiding tot arts-microbioloog.

Tijdens haar studie leerde zij haar man kennen, met wie zij trouwde in de zomer van 2000. Op dit moment wonen zij in Groningen stad samen met hun 2 dochters, Ruth (2004) en Hannah (2007).

List of Abbreviations

Ad	adenovirus
AIDS	acquired immune deficiency syndrome
APC	antigen-presenting cell
BHK	baby hamster kidney cells
BSA	bovine serum albumin
C	capsid protein
$C_{12}E_8$	octa(ethyleneglycol)-n-dodecyl monoether
CAR	coxsackie-adenovirus receptor
CD	cluster of differentiation
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
Cr	chromium
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
ds	double-stranded
E6	early protein 6 of HPV
E7	early protein 7 of HPV
ELISA	enzyme-linked immunosorbent assay
Elispot	enzyme-linked immunosorbent spot
e	enhancer
enh	enhancer
E:T ratio	effector cell to target cell ratio
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMDV	foot and mouth disease virus
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesolfonic acid]
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HNE	5 mM Hepes, 150mM NaCl and o,1 mM EDTA buffer
HPV	human papillomavirus
IgG	immunoglobulin G
IL	interleukine
i.m.	intramuscular
INFγ	interferon gamma
i.p.	intraperitoneal
IPTG	isopropyl-β-D-thiogalactopyranoside
i.v.	intravenous
L	late viral capsid protein of HPV
lacZ	β-galactosidase
LC	Langerhans cells
LPS	lipopolysaccharide
luc	luciferase
K10	keratine 10 promotor
MHC	major histocompatibility complex
mRNA	messenger RNA
NaCl	natriumchloride
NK	natural killer cells
nsP	non-structural proteins

ORF	open reading frame
OVA	chicken ovalbumin
p53	tumor protein 53
Pap	Papanicolaou
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pCTL	precursor cytotoxic T lymphocyte
pRb	retinoblastoma protein
rAd	recombinant adenovirus
RAHYNIVTF	HPV16 E7 CTL epitope, amino acid 49-57
RNA	ribonucleic acid
rSFV	recombinant Semliki Forest virus
s.c.	subcutaneous
SFV	Semliki Forest virus
SIL	squamous intraepithelial lesion
SIN	Sindbis virus
T_{CM}	central memory T cells
TCR	T cell receptor
T_{EM}	effector memory T cells
TGF-β	transforming growth factor beta
Th	T-helper cells
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cells
VEE	Venezuelan equine encephalitits virus
VIN	vulval intraepithelial neoplasia
VLP	virus-like particle
WHO	World Health Organisation